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PROVISIONAL SPECIFICATION

Invention Title: COMPARATIVE GENOMIC HYBRIDIZATION

Applicant: THE UNIVERSITY OF ADELAIDE

The invention is described in the following statement:

COMPARATIVE GENOMIC HYBRIDIZATION

Field of the Invention

The present invention relates to methods of comparative genomic hybridisation and to nucleic acids attached to a solid substrate suitable for comparative genomic hybridisation.

Background of the Invention

Chromosome abnormalities are often associated with genetic disorders, degenerative diseases, and cancer. Chromosomal abnormalities can be of several types, including extra or missing individual chromosomes, extra or missing portions of a chromosome, breaks and chromosomal rearrangements. Chromosomal rearrangements include translocations (transfer of a piece from one chromosome onto another chromosome), dicentrics (chromosomes with two centromeres), inversions (reversal in polarity of a chromosomal segment), insertions, amplifications, and deletions.

The detection of chromosomal abnormalities in cells is important for many reasons, not the least being the detection of chromosomal abnormalities for prenatal and pre-implantation genetic diagnosis, and the determination of the karyotype of some cancers.

Prenatal diagnosis involves the genetic testing of foetal material. Typically, this involves removal of amniotic fluid surrounding the foetus and the analysis of cells in the fluid for chromosomal abnormalities. Prenatal diagnosis is important for the detection of foetuses that have significant chromosomal errors. Detectable chromosomal abnormalities occur with a frequency of approximately one in every 250 human births and abnormalities that involve deletions or additions of chromosomal material often lead to foetal death or to serious

mental and physical defects. For example, Down Syndrome can be caused by having three copies of chromosome 21 instead of the normal two copies, or by a segmental duplication of a subregion on chromosome 21.

Pre-implantation genetic diagnosis (PGD) involves the testing of genetic material from an embryo or an egg (oocyte) prior to implanatation. Typically, this process involves the removal and analysis of one or more cells from an embryo fertilized *in vitro*, in order to determine if the embryo is suitable for implantation. In the case of maternally derived chromosomal abnormalities, the polar body from an oocyte can also be removed and the presence of a chromosomal abnormality detected.

Pre-implantation genetic diagnosis for chromosomal abnormalities is important for detecting embryos or oocytes that are suitable for implantation. Early human embryos have a very high frequency of chromosomal errors including aneuploidy, polyploidy and mosaicism, and it is likely that these chromosomal errors are responsible for the significant rate of implantation failure of *in vitro* fertilized embryos. In addition, where there is a possibility that an embryo or oocyte may contain a known chromosomal abnormality inherited from one of the parents, pre-implantation diagnosis can also be performed to select embryos or oocytes that do not have the known chromosomal abnormality.

The deletion or multiplication of copies of whole chromosomes or chromosomal segments also often occurs in cancerous cells and in many cases these chromosomal abnormalities contribute to the cells acquiring a cancerous phenotype. The detection of such chromosomal abnormalities is not only important for understanding the genetic basis of how some cells progress from a non-cancerous state to a cancerous state, but in some cases may provide useful information as to the diagnosis and treatment of a specific cancer.

Traditionally, cytogenetic or fluorescence *in situ* hybridisation (FISH) techniques have been used for detecting chromosomal abnormalities. However, comparative genomic hybridization (CGH) now provides a powerful method to overcome many of the limitations of the traditional cytogenetic and FISH approaches. CGH involves the comparative, multi-colour hybridization of a reference nucleic acid population labelled in one fluorescent colour and a sample nucleic acid population labelled in a second fluorescent colour to all or part of a reference genome, such as a human metaphase chromosome spread. Comparison of the resulting fluorescence intensity at locations in the reference genome permits determination of the copy number of chromosomal sequences in the sample population.

Although CGH has provided an improvement over traditional cytogenetic and FISH technologies, there are still many deficiencies associated with CGH, including the length of time required to perform the analysis. For example, standard CGH with metaphase spreads takes at least 72 hours to complete, and if PGD is being performed on a single cell taken from an embryo, then embryo cryopreservation before implantation must be performed so as to allow sufficient time to complete the procedure.

The present invention relates to the identification of an improved method for performing comparative genomic hybridization and to nucleic acid arrays suitable for comparative genomic hybridization.

Throughout this specification reference may be made to documents for the purpose of describing various aspects of the invention. However, no admission is made that any reference cited in this specification constitutes prior art. In particular, it will be understood that the reference to any document herein does not constitute an admission that any of these documents forms part of the common general knowledge in the art in Australia or in any other country. The discussion of the references states what their authors assert, and the applicant

reserves the right to challenge the accuracy and pertinency of any of the documents cited herein.

Summary of the Invention

The present invention provides a method of comparing at least one chromosome or part thereof from a cell with a first karyotype with the corresponding chromosome or part thereof from a cell with a second karyotype, the method including the steps of:

(a) amplifying DNA from an isolated chromosome or part thereof;

(b) attaching the amplified DNA to a solid substrate;

(c) amplifying DNA from one or more cells with a first karyotype and amplifying DNA from one or more cells with a second karyotype;

(d) labelling the amplified DNA from the one or more cells with the first karyotype with a first label, and labelling the amplified DNA from the one or more cells with the second karyotype with a second label, wherein the first and second labels are detectably different;

(e) hybridizing the amplified and labelled DNA from the one or more cells with the first karyotype with the amplified DNA attached to the solid substrate, and hybridizing the amplified and labelled DNA from the one or more cells with the second karyotype with the amplified DNA attached to the solid substrate; and

(f) comparing the relative amount of first and second labels hybridized to the amplified DNA attached to the solid substrate.

The present invention also provides a method of detecting a chromosomal abnormality in a cell with an unknown karyotype, the method including the steps of:

(a) amplifying DNA from an isolated chromosome or part thereof;

(b) attaching the amplified DNA to a solid substrate;

(c) amplifying DNA from one or more cells with an unknown karyotype and amplifying DNA from one or more cells with a reference karyotype;

(d) labelling the amplified DNA from the one or more cells with the unknown karyotype with a first label, and labelling the amplified DNA from the one or more cells with the reference karyotype with a second label, wherein the first and second labels are detectably different;

(e) hybridizing the amplified and labelled DNA from the one or more cells with the unknown karyotype with the amplified DNA attached to the solid substrate, and hybridizing the amplified and labelled DNA from the one or more cells with the reference karyotype with the amplified DNA attached to the solid substrate; and

(f) detecting the presence of a chromosomal abnormality in the cell with the unknown karyotype by comparing the relative amount of the first label hybridised to the amplified DNA attached to the solid substrate to the amount of second label hybridised to the amplified DNA attached to the solid substrate.

The present invention also provides a nucleic acid attached to a solid substrate, wherein the nucleic acid is derived from an isolated chromosome or part thereof and the nucleic acid is depleted of repetitive sequences.

The present invention also provides an array of nucleic acids attached to a solid substrate, wherein each nucleic acid in the array is derived from an isolated chromosome or part thereof and each nucleic acid is depleted of repetitive sequences.

The present invention also provides a nucleic acid attached to a solid substrate, wherein the nucleic acid is the product of randomly primed amplification of an isolated chromosome or part thereof and the nucleic acid is depleted of one or more of repetitive sequences, non-chromosomal sequences or sequences that are over-represented due to amplification of the chromosome or part thereof.

The present invention further provides an array of nucleic acids attached to a solid substrate, wherein each nucleic acid in the array is the product of

randomly primed amplification of an isolated chromosome or part thereof and each nucleic acid is depleted of one or more of repetitive sequences, non-chromosomal sequences or sequences that are over-represented due to amplification of the chromosome or part thereof.

The present invention arises out of studies into the detection of trisomies 13 and 18 in single cells of amniocytes and lymphocytes. In particular, it has been surprisingly found that the detection of such trisomies in a single cell by comparative genomic hybridization may be markedly improved by replacing the metaphase spreads normally employed with the products of randomly primed amplification of an isolated chromosome or part thereof attached to a solid substrate. Comparative genomic hybridization performed in this way requires less time (approximately 30 hours in total) to perform than traditional methods using metaphase spreads.

Various terms that will be used throughout the specification have meanings that will be well understood by a skilled addressee. However, for ease of reference, some of these terms will now be defined.

The term "cell" as used throughout the specification is to be understood to not only encompass the normal meaning of the word, but also to include any cell derived body having one or more chromosomes (or a part thereof) therein. Examples of such cell derived bodies include the polar body associated with an unfertilised oocyte, a polar body extruded by an oocyte at the time of oocyte fertilization by a sperm, a nucleus isolated from a cell or part of a nucleus.

The term "karyotype" as used throughout the specification is to be understood to mean the chromosomal constitution of a cell, which may vary between individuals of a single species.

In this regard, the term "unknown karyotype" is to be understood to mean that the karyotype of one or more chromosomes in a cell is not known. The term "reference karyotype" is to be understood to mean the karyotype of a cell that is used as the karyotype against which the karyotype of another cell is tested. The cell with the reference karyotype may have a known karyotype, such as a normal karyotype or a known deletion or multiplication of a specific chromosome, or alternatively may have an unknown karyotype of one or more chromosomes. Typically, the cell with an unknown karyotype will be a cell from a foetus, embryo, oocyte or cancer cell, and the cell with the reference karyotype will be the same type of cell or a similar cell with a normal karyotype.

The term "chromosome or part thereof" as used throughout the specification is to be understood to mean an entire chromosome or any part of an entire chromosome. In this regard, it is to be understood that part of a chromosome will include a part of a chromosome, isolated for example by microdissection or by flow cytometry, or any clone containing chromosomal (genomic) DNA. Examples of such clones include BAC, YAC and P1 clones containing genomic DNA, or any other clone having genomic DNA cloned into a suitable vector.

In this regard, it is also to be understood that the term "chromosome" means any chromosome present in a cell of any ploidy (haploid, diploid or polyploid), including a sex chromosome, an autosome, a mitochondrial chromosome, a chloroplast chromosome or an episome.

The term "amplifying" or variants thereof as used throughout the specification is to be understood to mean the production of additional copies of a nucleic acid sequence. For example, amplification may be achieved using polymerase chain reaction (PCR) technologies (essentially as described in Dieffenbach, C. W. and G. S. Dveksler (1995) PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y.) or by other methods of amplification, such as rolling circle amplification on circular templates, such as described in Fire, A. and Xu, S-Q. (1995) *Proc. Natl. Acad. Sci* 92:4641-4645.

In this regard, the term "randomly primed amplification" will be understood to mean amplification utilising one or more primers that results in amplification of substantially the entire target. For example, random amplification may be achieved with the use of one or more primers including a sequence of one or more random nucleotides, the sequence of random nucleotides being sufficiently long so as to enable the primer to hybridize to the target nucleic acid under selected conditions at random positions and serve as a primer for extension by a polymerase. For example, the primer may be a primer including a stretch of six or more contiguous nucleotides of random sequence. Alternatively, random amplification may be achieved with one or more primers of fixed sequence, but with the stringency of the amplification reaction sufficiently low to enable random amplification of the target.

In addition, it will be appreciated that amplification of DNA from one or more cells not only includes the amplification of the entire chromosomal content of one or more cells, but also includes the amplification of an isolated chromosome, or any part thereof, derived from one or more cells. For example, the DNA amplified for analysis could be a single chromosome isolated by microdissection or flow cytometry, a part of a chromosome isolated by microdissection, or part of a chromosome being a cloned fragment of genomic DNA.

The term "attaching" or variants thereof as used throughout the specification in relation to amplified DNA is to be understood to mean any form of immobilising amplified DNA to a solid substrate, including passive adsorption to the solid substrate, covalent linkage of the DNA to the solid substrate through appropriate chemical groups, or the use of specific chemical groups with high affinity for each other that allow the DNA to be immobilised on the solid substrate (eg biotin and streptavidin).

The term "solid substrate" as used throughout the specification is to be understood to mean any solid support that allows a nucleic acid to be spatially fixed to the support and which allows the nucleic acid to remain fixed to the

support during hybridisation. Examples of solid supports include glass, nylon or other type of membranes, filters, and chips.

In this regard, it will be understood that the nucleic acid need not be permanently fixed to the support, and that the nucleic acid may, if so desired, be fixed to the solid support so as to allow the removal of the nucleic acid from the support under selected conditions.

The term "chromosomal abnormality" as used throughout the specification is to be understood to mean any change or alteration in a part of a chromosome that may be detected by a method involving comparative genomic hybridization.

The term "germ cell" as used throughout the specification is to be understood to mean a reproductive cell, a gamete, or a cell that will develop into a reproductive cell. For example, a germ cell includes a spermatocyte or an oocyte.

The term "repetitive sequences" as used throughout the specification is to be understood to mean any sequence present in a nucleic acid that is present in more than one copy in the genome. Each copy of a repetitive sequence need not be identical to all the others, as long as the sequences are sufficiently similar that under the hybridization conditions being used the same fragment of probe nucleic acid is capable of forming stable hybrids with each copy. Examples of repetitive sequences include simple repeated DNA (eg Alu or Kpn elements), satellite repeats, mini-satellite repeats, chromosome-specific repeats, micro-satellite repeats, repeated genes (eg rRNA genes), sequences derived from transposable elements (eg transposons with DNA or RNA intermediates), elements derived from multiple copies of viruses such as retroviruses, repeats associated with centromeres or telomeres, or repeats associated with heterochromatin.

The term "nucleic acid" as used throughout the specification is to be understood to mean a polynucleotide or oligonucleotide, being composed of

deoxyribonucleotides or ribonucleotides in either single- or double-stranded form, including known analogues of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

The term "non-chromosomal sequences" as used throughout the specification is to be understood to mean any sequence in a nucleic acid sample that is not normally present in the nucleotide sequence of the genome of the nucleic acid sample, or the nucleotide sequence of one or more chromosomes or part thereof. Examples of such non-chromosomal sequences include sequences derived from a vector or plasmid, or contaminating sequences that may be present in a nucleic acid sample, such as sequences derived from *E.coli*.

The phrase "sequences that are over-represented due to amplification of the chromosome or part thereof" or the term "over-represented sequences" as used throughout the specification is to be understood to mean those sequences present after amplification of the target that have been disproportionately amplified in comparison to other sequences normally present in the target.

Brief Description of the Figures

Figure 1 shows electrophoresis of re-amplified DNA libraries of human chromosomes (autosomes 1-22; sex chromosomes X, Y) as described in Example 2. Size markers (λ *Hind*III, Puc19 *Hpa*II) are also shown.

Figure 2 shows FISH results of reamplified DNA libraries of human chromosomes, as described in Example 3. FISH was carried out using metaphase chromosome spreads of lymphocytes from normal male peripheral blood. Either SpectrumGreen-dUTP or SpectrumRed-dUTP was used to label DNA probes by DOP-PCR amplification. Except for very faint signals for the DNA library of chromosome 21, all the others uniformly painted their whole target chromosomes or q arms.

Figure 3 shows a depiction of the expected DNA array format by using loading of a 384-well-plate, as described in Example 4.

Figure 4 shows results of electrophoresis of re-amplified DNA libraries before, during and after size selection, as described in Example 4(ii) for the manufacture of second generation arrays.

Figure 5 shows the image and graphical representation of the data for the analysis of 47, XX, +13(Green) cell versus a 46, XY(Red) cell as described in Example 10.

Figure 6 shows the image and graphical representation of the data for the analysis of a 47, XY, +18 cell versus a 46, XX cell as described in Example 10.

Figure 7 shows one of the images which is representative of the experiments described in Example 11.

Figure 8 shows the image for the experiment of the analysis of a single cell with karyotype 47, XY, +18 versus a 46, XX single cell as described in Example 12.

General Description of the Invention

As mentioned above, in one form the present invention provides a method of comparing at least one chromosome or part thereof from a cell with a first karyotype with the corresponding chromosome or part thereof from a cell with a second karyotype, the method including the steps of:

(a) amplifying DNA from an isolated chromosome or part thereof;

(b) attaching the amplified DNA to a solid substrate;

(c) amplifying DNA from one or more cells with a first karyotype and amplifying DNA from one or more cells with a second karyotype;

(d) labelling the amplified DNA from the one or more cells with the first karyotype with a first label, and labelling the amplified DNA from the

one or more cells with the second karyotype with a second label, wherein the first and second labels are detectably different;

(e) hybridizing the amplified and labelled DNA from the one or more cells with the first karyotype with the amplified DNA attached to the solid substrate, and hybridizing the amplified and labelled DNA from the one or more cells with the second karyotype with the amplified DNA attached to the solid substrate; and

(f) comparing the relative amount of first and second labels hybridized to the amplified DNA attached to the solid substrate.

This form of the present invention allows an assessment of the number of copies of one or more chromosomes, or a region of one or more chromosomes, in a cell with a first karyotype to be compared to a corresponding chromosome or region in a cell with a second karyotype. As such, this form of the present invention may be used, for example, in prenatal genetic diagnosis, pre-implantation genetic diagnosis, gender determination or selection, or the determination of the karyotype of cancerous or other somatic cells.

Preferably, the cell with the first karyotype is of unknown karyotype, and the cell with the second karyotype is of known karyotype. For example, the cell with the first karyotype may have an unknown chromosomal abnormality at a particular chromosomal location, and the cell with the second karyotype may have a known normal chromosome at the corresponding chromosomal location.

This form of the present invention is useful for the detection of gross chromosomal differences in a cell, such as deletions, duplications or amplifications. Examples of conditions that may be amenable to detection by the present invention include Trisomy 21, 13 and 18 and the detection of missing chromosomes, such as occurs in Turner's syndrome (45, XO).

It will be appreciated that the present invention may be used to compare all chromosomes in a cell with a first karyotype with a cell with a second karyotype. However, depending on the amplified DNA being attached to the solid

substrate, the invention may also be used for comparing parts of one or more specific chromosomes between cells.

The chromosome to be compared may be any chromosome present in a cell of any ploidy (haploid, diploid or polyploid), including a sex chromosome, an autosome, a mitochondrial chromosome, a chloroplast chromosome or an episome. Preferably, the chromosome is a sex chromosome or an autosome. Most preferably, the chromosome is an autosome.

Similarly, the part of the chromosome to be compared may be part of any chromosome present in a cell of any ploidy (haploid, diploid or polyploid) including a sex chromosome, an autosome, a mitochondrial chromosome, a chloroplast chromosome or an episome. Preferably, the part of the chromosome is part of a sex chromosome or an autosome. Most preferably, the part of the chromosome is part of an autosome.

The cell with the first karyotype may be any cell for which the karyotype (first karyotype) is to be compared to the karyotype (second karyotype) of another cell. The cell with the first karyotype may be a eukaryotic or a prokaryotic cell. Preferably, the cell is a eukaryotic cell. More preferably, the cell is an animal or human cell. Most preferably, the cell is a human cell.

Preferably, the cell with the first karyotype is a foetal cell, a cell derived from an embryo, a germ cell, a cancerous cell or any other type of somatic cell with a karyotype to be compared to the karyotype of another cell. More preferably the cell with the first karyotype is a foetal cell, an embryonic cell or a germ cell. Most preferably, the cell is an embryonic cell or an oocyte.

Examples of a foetal cell include a foetal cell taken from the amniotic fluid surrounding the foetus, a foetal blood cell taken from the maternal circulation, or a foetal cell taken from the mother's reproductive tract (eg cervical or vaginal lavage). In this regard, foetal blood cells, unlike mature blood cells, are nucleated and can be isolated from the maternal circulation.

In the case of an embryonic cell, a small number of cells (usually one or two cells) may be removed from an embryo. In this procedure, one or more cells in an embryo may be removed by cleavage stage embryo biopsy. This procedure is usually performed on day 3 of development, when the embryo is at the 6-8 cell stage. The biopsy consists of two stages. The first is to make a hole in the zona pellucida that surrounds the embryo at this time, usually using acid Tyrodes solution or a non-contact laser. Once the hole is made, the cell may then be removed from the embryo.

In the case of a germ cell, for example an oocyte or sperm cell, the germ cell may be analysed directly. Alternatively, in the case of screening for maternal abnormalities, a polar body from the oocyte may be isolated.

In the case of a cancerous cell or any other somatic cell, one or more cells may be obtained from a subject by a suitable method known in the art, such as direct biopsy of cells or isolation from blood.

In this regard, a large number of cells may not necessarily be associated with a more accurate determination of karyotype, and in some cases it may be preferable that a relatively small number of cells (eg 1 to 20 cells) is isolated.

The cell with the second karyotype may be any cell for which the karyotype of the cell with the first karyotype is to be compared. Preferably, the cell with the second karyotype is of the same type or a similar type as the cell with the first karyotype.

The isolated chromosome in the various forms of the present invention may be any chromosome that has been substantially purified from other chromosomes by a method known in the art. For example, the chromosome may be isolated by microdissection as described in Meltzer *et al.* (1992) *Nature Genetics* 1:24-

28. Alternatively, the chromosome may be isolated by flow cytometry as described in Telenius *et al.* (1992) *Genes, Chromosomes & Cancer* 4:257-263.

In the case of microdissection, cells are first treated to force them into metaphase and an entire chromosome may be isolated with the use of a very fine needle. In the case of flow cytometry, chromosomes may be stained with specific chromosomal staining reagents and the chromosomes isolated by the extent of fluorescence associated with each of the chromosomes via sorting.

A part of a chromosome in the various forms of the present invention may be any part of a chromosome for which the karyotype is to be compared with the corresponding part of the same chromosome in another cell. A part of a chromosome may be isolated by a suitable method known in the art, including the microdissection of specific chromosomal bands from metaphase chromosomes as discussed above.

Alternatively, a part of a chromosome may be isolated by cloning a fragment of genomic DNA into a suitable vector. Methods for the isolation of large and small genomic fragments and their cloning into vectors are essentially as described in Sambrook, J, Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual 2nd. ed. Cold Spring Harbor Laboratory Press, New York. (1989). For example, to produce large genomic fragments for cloning into a vector such as a YAC, partial digestion of genomic DNA with a restriction endonuclease may be performed and the resulting fragments cloned into the vector. The isolated vector with cloned insert DNA may then be purified by a suitable method known in the art. Examples of suitable vectors for cloning large genomic fragments are YAC vectors, BAC vectors, P1 vectors or cosmids.

Amplification of the DNA from an isolated chromosome or part thereof in the various forms of the present invention may be achieved by a suitable method known in the art that allows the production of additional copies of the DNA. For example, in the case of an entire chromosome isolated by microdissection or flow cytometry, or a part thereof isolated by microdissection, the DNA may be

amplified using PCR technology, essentially as described in Dieffenbach, C. W. and G. S. Dveksler (1995) PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y.

In the case of part of a chromosome cloned into a circular vector, amplification may be achieved for example by PCR amplification or by the use of rolling circle amplification as described in Fire, A. and Xu, S-Q. (1995) *Proc. Natl. Acad. Sci* 92:4641-4645.

Preferably the amplification of the DNA from an isolated chromosome or part thereof will result in the amplification of substantially the entire target.

Accordingly, it is preferred that the amplification of the isolated chromosome or a part thereof is randomly primed amplification to achieve amplification of substantially the entire target.

Amplification of the DNA from an isolated chromosome or part there of will be performed with one or more appropriate primers. As discussed above, preferably the one or more primers used will result in the random amplification of the isolated chromosome or part thereof.

Preferably, the one or more primers used is an oligonucleotide including one or more nucleotides of random sequence. More preferably, the one or more primers is an oligonucleotide including one or more contiguous nucleotides of random sequence. More preferably, the one or more of primers is an oligonucleotide that includes six or more contiguous nucleotides of random sequence, such as a DOP primer (degenerate oligonucleotide primer). Most preferably, the one or more primers is a primer with the following nucleotide sequence:

5'-CCGACTCGAGNNNNNNATGTGG-3' (SEQ ID NO. 1);

where NNNNNN represents the degenerate sequence. "N" represents the four possible nucleotides in the DNA sequence: "A", "T", "C" and "G" for Adenine, Thymine, Cytosine and Guanine, respectively. As such, the degenerate sequence contain mixtures of various nucleotide sequences including all possible combinations of A, T, C and G at the "N" positions.

If so desired, the nucleotide sequence of the degenerate sequence can also be biased towards a particular nucleotide composition, for example GC or AT richness.

In the case of amplification using DOP primers on an isolated chromosome, or a part of a chromosome isolated by microdissection or by cloning into a vector, the amplification may be performed essentially as described in Telenius *et al.* (1992) *Genomics* 18:718-725 (1992). Briefly, the amplification is performed under low stringency conditions for a low number of cycles (eg five cycles) and a second stage amplification performed under more stringent conditions for a larger number of cycles (eg 35 cycles).

Alternatively, random primed amplification may be achieved using one or more primers of fixed sequence and performing a low number of cycles of amplifcation under low stringency conditions that allow the one or more primers to prime synthesis randomly throughout the target, followed by a second stage amplification performed under more stringent conditions for generally a larger number of cycles.

In addition, to account for regions of small chromosomes that may present a difficulty in achieving randomly primed amplification of substantially the entire target, region specific primers can also be used in conjunction with other primers that allow random amplification. For example, primers to specific regions of chromosomes 21 and 22 may be used in conjunctions with DOP primers.

Other suitable techniques for amplification of the isolated chromosome or part thereof include primer-extension pre-amplification PCR (PEP-PCR) which may be performed essentially as described in Zhang *et al.* (1992) *Proc Natl. Acad. Sci* 89:5847-5851); ligation mediated PCR, which may be performed essentially as described in Klein *et al.* (1999) *Proc. Natl. Acad. Sci.* 96:4494-4499); or alu-PCR, which may be performed essentially as in Nelson *et al.* (1989) *Proc. Natl. Acad. Sci.* 86:6686-6690).

In the case of using rolling circle amplification on a cloned genomic insert in a circular vector, rolling circle amplification may be performed using suitable conditions known in the art, for example as described in Fire, A. and Xu, S-Q. (1995) *Proc. Natl. Acad. Sci* 92:4641-4645.

Preferably, the amplified DNA from the isolated chromosome or part thereof is further subjected to size selection before attachment to the solid substrate. Preferably, the amplified DNA attached to the solid substrate is less than 10 kb in size. More preferably, the amplified DNA attached to the solid substrate is less than 3 kb in size.

Size selection may be performed by a suitable method known in the art. For example, the amplified DNA may be electrophoresed on an agarose gel, and DNAs with a size in the range from 150 to 3000 bp may be isolated.

In a preferred form, the nucleic acid attached to the solid substrate is the product of randomly primed amplification of an isolated chromosome or part thereof, wherein the nucleic acid has been size selected. In this case, the randomly amplified DNA attached to the solid substrate is preferably less than 10 kb in size. More preferably, the randomly amplified DNA attached to the solid substrate is less than 3 kb in size. For example, the randomly amplified DNA may be electrophoresed on an agarose gel, and DNAs with a size in the range from 150 to 3000 bp may be isolated.

In a further preferred embodiment, the amplified DNA from the isolated chromosome or part thereof is depleted of one or more of repetitive sequences, non-chromosomal sequences, or sequences that are over-represented due to amplification. A number of methods known in the art can be used to deplete the amplified DNA of such sequences.

Repetitive sequences are sequences present in more than one copy in the target sequence to be amplified. Non-chromosomal sequences are sequences that are not normally present in the nucleotide sequence of the chromosome or part thereof, such as sequences derived from a vector or plasmid, or contaminating sequences that may be present in the originally target sample to be amplified, such as sequences derived from *E.coli*. Sequences that are over-represented due to amplification of the chromosome are those sequences present after amplification of the target that have been disproportionately amplified in comparison to other sequences normally present in the target.

Repetitive sequences and/or non-chromosomal sequences may be removed either prior to amplification or after amplification. For example, chromosomal DNA may be isolated and repetitive sequences and/or non-chromosomal sequences removed. Alternatively, the DNA may first be amplified with appropriate primers and the repetitive DNA sequences and/or non-chromosomal sequences removed from the amplified pool of nucleic acids.

Examples of repetitive sequences include simple repeated DNA (eg Alu or Kpn elements), satellite repeats, mini-satellite repeats, chromosome-specific repeats, micro-satellite repeats, repeated genes (eg rRNA genes), sequences derived from transposable elements (eg transposons with DNA or RNA intermediates), elements derived from multiple copies of viruses such as retroviruses, repeats associated with centromeres or telomeres, or repeats associated with heterochromatin.

A number of methods known in the art can be used to remove repetitive sequences. For example, in many genomes, such as the human genome, a

major portion of repetitive DNA is contained in a few families of highly repeated sequences such as Alu. To remove such repetitive sequences, a blocking procedure can be used. These methods primarily exploit the fact that the hybridization rate of complementary nucleic acid strands increases as their concentration increases. Thus, if a mixture of nucleic acid fragments is denatured and incubated under conditions that permit hybridization, the sequences present at high concentration will become double-stranded more rapidly than the others. The double-stranded nucleic acid can then be removed by the direct removal of these sequences by a method known in the art.

For example, single- and double-stranded nucleic acids have different binding characteristics to hydroxyapatite. Such characteristics provide a basis commonly used for fractionating nucleic acids. The fraction of genomic DNA containing sequences with a particular degree of repetition can be obtained by denaturing genomic DNA, allowing it to reassociate under appropriate conditions, followed by separation using hydroxyapatite. Such techniques are as described in Britten *et al.*, "Analysis of Repeating DNA Sequences by Reassociation" *Methods in Enzymology* 22: 363-418 (1974).

Examples of such sequences that can be used to deplete the amplified DNA of repetitive sequences include human Cot-1 DNA and Alu – repeat containing DNAs.

Alternatively, reaction with immobilized nucleic acid may be performed. For example, minimally sheared human genomic DNA is bound to diazonium cellulose or a like support. The amplified DNA, appropriately cut into fragments, is hybridized against the immobilized DNA to Cot values in the range of about 1 to 100. The material that does not bind to the immobilised nucleic acid may then be attached to the solid substrate.

In the case of repetitive sequences depleted from a genomic clone, the repetitive sequences may be depleted from a clone of genomic DNA (eg

removed during the cloning process) and the resulting clone depleted of repetitive sequences used for amplification.

Similarly, non-chromosomal sequences may be depleted before or after amplification of the target sequence. In a similar manner to as described above for the depletion of repetitive sequences, non-chromosomal sequences may be depleted by using non-chromosomal sequences in excess in a hybridisation reaction with the target or the amplified DNA, or by attaching the non-chromosomal sequences to a solid support and using these sequences to deplete the DNA of these sequences.

In the case of depleting over-represented sequences due to amplification, these sequences may be depleted from the target before amplification by similar methods described above, or the over-represented sequences may be depleted after amplification. As will be appreciated, an understanding of the actual sequence being over-represented is necessary, and will depend on the primers being used and the nature of the target being amplified.

The solid substrate in the various forms of the present invention is any solid support that allows a nucleic acid to be spatially fixed to the support and which allows the nucleic acid to remain fixed to the support during and after hybridisation. Examples of solid supports include glass, nylon or other type of membranes, filters, and chips.

The amplified DNA may be attached to a solid substrate in the various forms of the present invention by a suitable method known in the art, including passive adsorption or covalent linkage. For example, the amplified DNA may be attached to a glass substrate by passive adsorption by spotting samples onto a Polysine™ microscope glass slide (Menzel-Glaser, Gemany) and processing of the slide by dehydration, snap-drying, fixation through UV cross linking, and chemical blocking by using succinic anhydride. In the case of covalent linkage, the amplified DNA may be attached to the solid substrate by a suitable method known in the art.

It is preferred that more than one amplified DNA is attached to the solid substrate, to produce an array of deposited DNAs. Such an array can be manufactured in any desired manner known in the art, including robotic deposition of the amplified DNAs. Examples of methods for producing arrays are essentially as described in U.S. Patents 5,486,452, 5,830,645, 5,807,552, 5,800,992 and 5,445,934.

Any suitable amount of DNA may be deposited on the solid substrate. The amount of nucleic acid deposited can be from about 0.05 nl to about 5.0 nl of a nucleic acid solution of 0.15 - 1 μg/μl nucleic acid concentration. For example, for a density of 1,000 DNAs deposited/cm, the individual amount deposited is about 0.2 nl to about 2.0 nl of 1 μg/μl solution. The DNA is provided in any solvent that will permit deposition of the nucleic acid.

The array having deposited DNAs may be produced in any arrangement. For example, the DNAs can be located in one portion of the array or can be interspersed among other deposited nucleic acids. The regularity of a two dimensional array is preferred.

It is also preferred that the array include various control nucleic acids, such as, for example, spotted nucleic acids of known copy number for a particular expressed gene or genomic sequence. For example, genomic DNA extracted from cell lines with 1 or more copies of a particular chromosome can be used, or the entire DOP-PCR products of amplification of DNA from a single cell can also be used.

The number of cells to be analysed in this form of the present invention is not particularly limited, and may range from a single cell (for example isolated from an embryo) to a large number of cells (for example isolated from a tissue biopsy or blood). For example, the method of the present invention may be applied to PGD on a single cell isolated from an embryo or a polar body from an oocyte, prenatal diagnosis of foetal cells, or the determination of the karyotype of

cancer cells or other somatic cells isolated from a subject by biopsy or isolated from the blood.

In a preferred form of the invention, the cell to be analysed is a single cell or a small number of cells, being in the range from 2 to 20 cells.

Preferably, the number of cells from which DNA is to be extracted and amplified is the same or similar between the cell or cells with the first karyotype and the cell or cells with the second karyotype. For example, in the case of PGD on a single cell from an embryo or the polar body from an oocyte, a single cell from another source will be preferably used for the comparison.

To obtain DNA from the cell for amplification in the various forms of the present invention, a suitable method known in the art for lysing the cell and obtaining the DNA may be used. For example, treatment of a cell with a hydroxide solution and subsequent neutralization lyses the cell and allows the extracted DNA to be directly amplified.

Preferably the amplification will result in the amplification of substantially the entire extracted DNA. Accordingly, it is preferred that the amplification of the DNA is randomly primed amplification.

Amplification of the DNA from the cell in the various forms of the present invention will be performed with one or more appropriate primers. As discussed above, preferably the one or more primers used will result in the random amplification of the DNA.

To amplify DNA from one or more cells, the amplification of the extracted DNA may then be performed with one or more appropriate primers by a suitable method known in the art, such as PCR.

Preferably, the one or more of the primers used for amplification is an oligonucleotide including one or more nucleotides of random sequence. More

preferably, the one or more primers is an oligonucleotide including one or more contiguous nucleotides of random sequence. More preferably, the one or more of the primers is an oligonucleotides that includes six or more contiguous nucleotides of random sequence, such as a DOP primer (degenerate oligonucleotide primer). Most preferably, the one or more primers is a primer with the following nucleotide sequence:

5'-CCGACTCGAGNNNNNNATGTGG-3' (SEQ ID NO. 1);

where NNNNNN represents the degenerate sequence. "N" represents the four possible nucleotides in the DNA sequence: "A", "T", "C" and "G" for Adenine, Thymine, Cytosine and Guanine, respectively. As such, the degenerate probe sequences contain mixtures of various probes including all possible combinations of A, T, C and G at the "N" positions.

If so desired, the nucleotide sequence of the degenerate sequence can also be biased towards a particular nucleotide composition, for example GC or AT richness.

In the case of amplification using DOP primers, the amplification may be performed essentially as described in Telenius *et al.* (1992) *Genomics* 18:718-725 (1982). Briefly, the amplification is performed under low stringency conditions for a low number of cycles (eg five cycles) and a second stage amplification performed under more stringent conditions for a larger number of cycles (eg 35 cycles).

Alternatively, random primed amplification may be achieved using one or more primers of fixed sequence and performing a low number of cycles of amplifcation under low stringency conditions that allow the one or more primers to prime synthesis randomly throughout the target, followed by a second stage amplification performed under more stringent conditions for a larger number of cycles.

Other suitable techniques for amplification of the extracted DNA include primer-extension pre-amplification PCR (PEP-PCR) which may be performed essentially as described in Zhang *et al.* (1992) *Proc Natl. Acad. Sci* 89:5847-5851); ligation mediated PCR, which may be performed essentially as described in Klein *et al.* (1999) *Proc. Natl. Acad. Sci.* 96:4494-4499); or alu-PCR, which may be performed essentially as in Nelson *et al.* (1989) *Proc. Natl. Acad. Sci.* 86:6686-6690).

Amplification may be performed under suitable conditions known in the art. For example, for the amplification by PCR of genomic DNA isolated from a single lymphocyte cell isolated from blood, lysis of the single cell may be achieved by treatment with a lysis buffer (200mM KOH, 50mM dithiothreitol) for 10 min at 65°C followed by neutralization with 300mM KCl, 900mM Tris-HCl, Ph 8.3, 200mM HCl. To the lysed and neutralized solution may be added an appropriate PCR buffer, Taq polymerase and amplification performed using an initial denaturation step of 95°C for 5 min and subsequent cycling conditions of 8 cycles of low stringent amplification of 94°C for 1 min, 30°C for 1.5 min, 72°C for 3 min with a ramp of 1°C per 4 seconds for increasing temperature from 30°C to 72°C, followed by 26 cycles of high stringent amplification of 94°C for 1 min, 62°C for 1 min, 72°C form 3 min with an addition of 14 seconds per cycle to the extension step. An extension step of 72°C for 10 min may then be performed to complete the amplification.

Preferably, extracted DNA from the cell with the first karyotype and extracted DNA from the cell with the second karyotype are amplified with the same primers, and preferably also under the same conditions. In this way, the quality and quantity of the extension products resulting from the amplification reaction are comparable.

It may also be desired to compare a specific chromosomal region or gene in the cell with a first karyotype with the same region or gene in the cell with a second karyotype.

The specific primer or the set of specific primers may be added to other primers that are present in the reaction mix for the purposes of, for example, randomly amplifying the extracted genomic DNA. Alternatively, the specific primer or the set of specific primers may be used as the only primers to amplify the extracted DNA.

Examples of primers that may be used are primers that amplify a specific region of a chromosome, such as primers to small chromosomes (eg chromosomes 21 and 22). In such a case, it may be preferable to add such primers to a set of primers that randomly amplify the genomic DNA, for example DOP primers.

In the situation where it is desired to amplify a specific chromosomal region, a specific locus, or one or more specific genes, primers to the particular region may be used alone or in combination with other primers, such as DOP primers to randomly amplify genomic DNA. For example, chromosomal regions including the regions involved in diseases such as thalassemia, Duchenne muscular dystrophy, X-linked disorders and Haemophilia may be amplified for analysis of chromosomal abnormalities. It will be appreciated in this regard that the method of the current invention is useful for the detection of major chromosomal abnormalities such as deletions and multiplications, and the specific loci being amplified will need to carry such abnormalities to allow their detection by the method of the present invention.

Suitable appropriate primers for amplification of specific chromosomal regions may be identified from the known nucleotide sequence. In the case of the amplification of specific chromosomal regions in humans, appropriate primers may be selected by consideration of the commercial Celera nucleotide sequence database or the publicly accessible nucleotide sequence database available from NCBI.

For example, exon 11 of the cystic fibrosis gene (CFTR) may be amplified using a nested PCR approach. For the first round, the primers 5'-TGAAATAATGGAGATGCAATGTTC-3' and

5'GCACAGATTCTGAGTAACCATAAT3' can be used. For the second round, 5'-CAACTGTGGTAAAGCAATAGTGT-3' and 5'-TACCAAATCTGGATACTATACCAT-3' are used as primers. Suitable amplification conditions for the first round include 1/10th of the DOP-PCR mix from the amplification of the DNA from a single cell, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 100 μM each dNTP, 2.5 mM $MgCl_2$ and 1U Taq polymerase, using the following conditions in a MJ Researcher PTC-100 PCR machine with hot bonnet: place reaction tubes into 96°C block and perform initial denaturation step of 94°C for 5 min, cycling conditions of 94°C for 30 sec, 62°C for 45 sec, 72°C for 45 sec, for 30 cycles. The second round PCR consists of 3 μl of the amplification products from the first round PCR, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 100 μM each dNTP, 2.5 mM $MgCl_2$ and 1U Taq polymerase. Cycling conditions use a MJ Researcher PTC-100 PCR machine with hot bonnet: and cycling conditions of 94°C for 30 sec, 52°C for 45 sec, 72°C for 45 sec, for 30 cycles. After amplification, products can be sequenced essentially as described Hussey *et. al.* (2002) *Mol. Hum. Reprod.* 8:1136-1143.

Labelling of amplified genomic DNA in the various forms of the present invention with a suitable label may be achieved by a suitable method known in the art. Labelling may occur after amplification, or alternatively, may occur during or as part of the initial amplification.

For example, direct labelling of the amplified genomic DNA described above may accomplished by further rounds of PCR incorporating a fluorescent moiety attached to a nucleotide to be incorporated. Other methods of indirect labelling are also known in the art. Alternatively, the amplified DNA may be labelled by subjecting the DNA to nick translation with a labelled nucleotide, essentially as described in Kirchhoff *et al.* (1998). *Cytometry* 31:163-173.

The amplified DNA from one or more cells with a first karyotype will be labelled with a first label, and the amplified DNA from one or more cells with a second karyotype will be a labelled with a second label that is detectably different from the first label. Examples of detectably different labels for incorporation into DNA

include SpectrumGreen-dUTP and SpectrumRed-dUTP (both from Vysis), or Cy3-dUTP and Cy5-dUTP.

For hybridisation of the amplified and labelled genomic DNAs to the DNA attached to the solid substrate in the various forms of the present invention, it is preferable that repetitive sequences, non-chromosomal sequences or sequences over-represented due to amplification, do not dominate the signal, and that they be depleted from the pool or that their ability to hybridize be suppressed as necessary.

Such sequences may be depleted either prior to amplification or after amplification. For example, genomic DNA may be extracted and repetitive sequences, non-chromosomal sequences or sequences over-represented due to amplification depleted. Alternatively, the genomic DNA may first be amplified with appropriate primers and then these sequences depleted from the amplified pool of nucleic acids.

A number of methods known in the art can be used to remove such sequences, and/or disable the hybridization capacity of such sequences.

For example, in many genomes, such as the human genome, a major portion of repetitive DNA is contained in a few families of highly repeated sequences such as Alu. To remove such repetitive sequences, a blocking procedure can be used. These methods primarily exploit the fact that the hybridization rate of complementary nucleic acid strands increases as their concentration increases. Thus, if a mixture of nucleic acid fragments is denatured and incubated under conditions that permit hybridization, the sequences present at high concentration will become double-stranded more rapidly than the others. The double-stranded nucleic acid can then be removed and the remainder used in the hybridization. A blocking method is generally described in the context of Southern analysis by Sealy *et al.*, "Removal of Repeat Sequences form Hybridization Probes", *Nucleic Acid Research 13*:1905 (1985).

Examples of such sequences that can be used to deplete the amplified DNA of repetitive sequences include human Cot-1 DNA and Alu – repeat containing DNAs.

Repetitive sequences may be depleted by the direct removal of these sequences by a method known in the art. For example, single- and double-stranded nucleic acids have different binding characteristics to hydroxyapatite. Such characteristics provide a basis commonly used for fractionating nucleic acids. The fraction of genomic DNA containing sequences with a particular degree of repetition can be obtained by denaturing genomic DNA, allowing it to reassociate under appropriate conditions, followed by separation using hydroxyapatite. Such techniques are as described in Britten *et al.*, "Analysis of Repeating DNA Sequences by Reassociation" *Methods in Enzymology* 22: 363-418 (1974).

Alternatively, the reaction with immobilized nucleic acid may be performed. For example, minimally sheared human genomic DNA is bound to diazonium cellulose or a like support. The amplified genomic DNA, appropriately cut into fragments, is hybridized against the immobilized DNA to Cot values in the range of about 1 to 100.

Non-chromosomal sequences or over-represented sequences may be removed by similar methods as described above, with the exception that vector and/or contaminating sequences are used to deplete the DNA of these sequences in the case of non-chromosomal sequences, and over-represented sequences used to deplete the DNA in the latter case.

Hybridization of the amplified and labelled DNA to the amplified DNA attached to the solid substrate in the various forms of the present invention may be performed by a suitable method known in the art. The hybridisation of the amplified DNA from the cell with the first karyotype to the DNA attached to the solid substrate, and the hybridisation of amplified DNA from the cell with the second karyotype to the DNA attached to the solid substrate, may be performed concurrently or sequentially.

The DNA is hybridized to DNA attached to the solid substrate under appropriate conditions. The hybridization conditions include choice of buffer, denaturant, such as formamide, salt additives and accelerant. The buffer will preferably have a pH of about 6.8 to about 7.2, a salt content of about 1.5X SSC to about 2.5X SSC, and a formamide content of about 40-50%. Suitable conditions can include a temperature of about 40 to about 80 degrees centigrade for a time sufficient to detect signal over background for both genomic and expression of about 1 to about 72 hours, preferably 12-24 hours. Hybridization accelerators, such as dextran sulfate, can be used if desired. The post-hybridization wash is preferably at a stringency greater than that of the hybridization.

It is preferred that during hybridisation an excess of unlabeled human repeat sequence DNA, such as Cot-1 DNA is also added. Use of unlabelled repeat sequence DNA in the hybridisation mix is generally in amounts of about 0.01 to about 5.0 μg per ng of total labelled genomic DNA.

The hybridization can be performed in any suitable apparatus that will maintain the amplified and labelled DNA in contact with the DNA attached to the solid support.

After hybridization, fluorescence intensity for each label is detected and determined by any suitable detector or reader apparatus and method. Laser-based array scanning detectors are known in the art.

The imaging apparatus and method in the various forms of the present invention may employ digital image processing algorithms used in a programmed computer for data analysis, storage and display of digital image data from the imaging apparatus. Any suitable digital image processing, data storage and display software can be used for analysis of the hybridization results.

The fluorescent data at each target element can be compared automatically to produce the ratio between the detectably different labels used.

The comparison of the relative amount of the first and second labels hybridised to the amplified DNA attached to the solid substrate may be used to detect whether the cell with the first karyotype has the same karyotype at a particular chromosomal position as the cell with the second karyotype, or alternatively, whether the cell with the first karyotype has a different karyotype at a particular chromosomal position as the cell with the second karyotype.

For the cell with the first karyotype to have the same karyotype as a cell with the second karyotype at a particular chromosomal location, preferably the ratio of the first (eg green) and second (eg red) labels hybridised to the amplified DNA will be in the range of 0.80 (ie ratio of red/green) for an autosome or 0.75 (ie ratio of red/green) for a sex chromosome to 1.20 (ie ratio of red/green) for an autosome or 1.25 (ie ratio of red/green) for a sex chromosome.

Alternatively, for the cell with the first karyotype to be deficient in a copy of a region of a chromosome as compared to the cell with the second karyotype at a particular chromosomal location, preferably the ratio of the first (eg green) and second (eg red) labels hybridised to the amplified DNA will be larger than 1.20 (ie ratio of red/green) for an autosome or larger than 1.25 (ie ratio of red/green) for a sex chromosome. Conversely, for the cell with the first karyotype to have an additional copy of a region of a chromosome as compared to the cell with the second karyotype at a particular chromosomal location, preferably the ratio of the first (eg green) and second (eg red) labels hybridised to the amplified DNA will be less than 0.80 (ie ratio of red/green) for an autosome or less than 0.75 (ie ratio of red/green) for a sex chromosome.

The present invention also provides a method of detecting a chromosomal abnormality in a cell with an unknown karyotype, the method including the steps of:

(a) amplifying DNA from an isolated chromosome or part thereof;

(b) attaching the amplified DNA to a solid substrate;

(c) amplifying DNA from one or more cells with an unknown karyotype and amplifying DNA from one or more cells with a reference karyotype;

(d) labelling the amplified DNA from the one or more cells with the unknown karyotype with a first label, and labelling the amplified DNA from the one or more cells with the reference karyotype with a second label, wherein the first and second labels are detectably different;

(e) hybridizing the amplified and labelled DNA from the one or more cells with the unknown karyotype with the amplified DNA attached to the solid substrate, and hybridizing the amplified and labelled DNA from the one or more cells with the reference karyotype with the amplified DNA attached to the solid substrate; and

(f) detecting the presence of a chromosomal abnormality in the cell with the unknown karyotype by comparing the relative amount of the first label hybridised to the amplified DNA attached to the solid substrate to the amount of second label hybridised to the amplified DNA attached to the solid substrate.

The chromosomal abnormality may be any change or alteration in a chromosome that may be detected by a method utilising comparative genomic hybridization. Examples of chromosomal abnormalities that may be detected by this form of the present invention include extra or missing individual chromosomes, extra or missing portions of a chromosome, breaks and chromosomal rearrangements such as translocations, dicentrics, inversions, insertions, amplifications, and deletions.

The cell with the unknown karyotype may be any cell for which the presence of a chromosomal abnormality is to be screened. Examples of chromosomal abnormalities that may be amenable to detection by the present invention include Trisomy 21, 13 and 18 and the detection of missing chromosomes, such as occurs in Turner's syndrome (45, XO).

The chromosome abnormality may be associated with any chromosome present in a cell of any ploidy (haploid, diploid or polyploid), including a sex chromosome, an autosome, a mitochondrial chromosome, a chloroplast chromosome or an episome. Preferably, the chromosome abnormality is present on a sex chromosome or an autosome. Most preferably, the chromosome abnormality is present on an autosome.

The cell with the unknown karyotype may be a eukaryotic or a prokaryotic cell. Preferably, the cell is a eukaryotic cell. More preferably, the cell is an animal or human cell. Most preferably, the cell is a human cell.

Preferably, the cell with the unknown karyotype is a foetal cell, a cell derived from an embryo, a germ cell, a cancerous cell or any other type of somatic cell with a chromosomal abnormality to be screened. More preferably the cell with the unknown karyotype is a foetal cell, an embryonic cell or germ cell. Most preferably, the cell with the unknown karyotype is an embryonic cell or a germ cell.

Accordingly in a preferred form, the present invention also provides a method of detecting a chromosomal abnormality in an embryo or a germ cell, the method including the steps of:

(a) amplifying DNA from an isolated chromosome or part thereof;
(b) attaching the amplified DNA to a solid substrate;
(c) isolating a cell from an embryo or a germ cell;
(d) amplifying DNA from the cell isolated from an embryo or germ cell;
(e) amplifying DNA from one or more cells with a reference karyotype;
(f) labelling the amplified DNA from the cell isolated from an embryo or germ cell with a first label, and labelling the amplified DNA from the one or more cells with the reference karyotype with a second label, wherein the first and second labels are detectably different;
(g) hybridizing the amplified and labelled DNA from the cell isolated from an embryo or germ cell with the amplified DNA attached to the solid substrate, and hybridizing the amplified and labelled DNA from the

one or more cells with the reference karyotype with the amplified DNA attached to the solid substrate; and

(h) detecting the presence of a chromosomal abnormality in the embryo or the germ cell by comparing the relative amount of the first label hybridised to the amplified DNA attached to the solid substrate to the amount of second label hybridised to the amplified DNA attached to the solid substrate.

In detecting a chromosomal abnormality, a large number of cells may not necessarily be associated with a more accurate determination of the presence of a particular chromosomal abnormality, and in some cases it may be preferable that a relatively small number of cells is isolated.

The cell with the reference karyotype may be any cell for which the karyotype of the cell with the unknown karyotype is to be compared. Preferably, the cell with the reference karyotype is from the same species as the cell with the unknown karyotype and also of the same type or a similar type as the cell with the unknown karyotype.

The number of cells to be analysed in this form of the present invention is not particularly limited, and may range from a single cell (for example isolated from an embryo) to a large number of cells (for example isolated from a tissue biopsy or blood). For example, the method of the present invention may be applied to PGD on a single cell isolated from an embryo or a polar body from an oocyte, prenatal diagnosis of foetal cells, or the determination of the presence of a chromosomal abnormality in cancer cells or other somatic cells isolated from a subject by biopsy or isolated from the blood.

In a preferred form of the invention, the cell to be analysed is a single cell or a small number of cells, being in the range from 2 to 20 cells.

Preferably, the number of cells from which DNA is to be extracted and amplified is the same or similar between the cell or cells with the unknown karyotype and

the cell or cells with the reference karyotype. For example, in the case of PGD on a single cell from an embryo or the polar body from an oocyte, a single cell from another source will be preferably used for the comparison.

In one embodiment, it may also be desired to detect a specific chromosomal abnormality in the cell with an unknown karyotype with the same region in a cell with a reference karyotype. In this form, a specific primer or a set of specific primers may be added to the extracted DNAs from both the cell with the unknown karyotype and the cell with the reference region.

The specific primer or the set of specific primers may be added to other primers that are present in the reaction mix for the purposes of, for example, randomly amplifying the extracted genomic DNA. Alternatively, the specific primer or the set of specific primers may be used as the only primers to amplify the extracted DNA.

Examples of primers that may be used to detect a chromosomal abnormality in a specific region are primers that amplify a specific region of a small chromosome (eg chromosomes 21 and 22). In such a case, it may be preferable to add such primers to a set of primers that randomly amplify the genomic DNA, such as DOP primers.

In the situation where it is desired to amplify a specific chromosomal region, a specific locus, or one or more specific genes associated with a chromosomal abnormailty, primers to the particular region may be used alone or in combination with other primers, such as DOP primers to randomly amplify genomic DNA. For example, chromosomal regions including the regions involved in diseases such as thalassemia, Duchenne muscular dystrophy, X-linked disorders and Haemophilia may be amplified for analysis of chromosomal abnormalities. It will be appreciated in this regard that the method of the current invention is useful for the detection of major chromosomal abnormalities such as deletions and multiplications, and the specific loci being amplified will need to

carry such abnormalities to allow their detection by the method of the present invention.

The amplified DNA from the one or more cells with the unknown karyotype will be labelled with a first label, and the amplified DNA from one or more cells with the reference karyotype will be a labelled with a second label that is detectably different from the first label. Examples of detectably different labels for incorporation into DNA include SpectrumGreen-dUTP and SpectrumRed-dUTP (both from Vysis), or Cy3-dUTP and Cy5-dUTP.

The comparison of the relative amount of the first and second labels hybridised to the amplified DNA attached to the solid substrate may be used to detect whether the cell with the unknown karyotype has a chromosomal abnormality at a particular chromosomal position as the cell with the reference karyotype.

For the detection of a deficiency in a copy of a region of a chromosome, preferably the ratio of the first (eg green) and second (eg red) labels hybridised to the amplified DNA will be larger than 1.20 (ie ratio of red/green) for an autosome or larger than 1.25 (ie ratio of red/green) for a sex chromosome. Conversely, for the detection of an additional copy of a region of a chromosome, preferably the ratio of the first (eg green) and second (eg red) labels hybridised to the amplified DNA will be less than 0.80 (ie ratio of red/green) for an autosome or less than 0.75 (ie ratio of red/green) for a sex chromosome.

The present invention also provides a nucleic acid attached to a solid substrate, wherein the nucleic acid is derived from an isolated chromosome or part thereof and the nucleic acid is depleted of repetitive sequences.

This form of the present invention provides a nucleic acid attached to a solid substrate that is useful not only for comparative genomic hybridisation, but is also useful as a substrate for detecting nucleic acids in any hybridisation based system.

The nucleic acid attached to the solid substrate may be any nucleic acid that is derived from an isolated chromosome or part thereof. The nucleic acid may be directly derived from the isolated chromosome or part thereof. For example, DNA from one or more isolated chromosomes may be directly attached to a solid substrate, or the DNA from one or more clones containing genomic DNA may be directly attached to the solid substrate.

Alternatively, the nucleic acid attached to the solid substrate may be the product of amplification of an isolated chromosome or part thereof.

Preferably, the nucleic acid attached to the solid substrate is the product of amplification of the DNA from an isolated chromosome or part thereof.

In the case of amplification of the DNA from an isolated chromosome or part thereof, preferably the amplification will result in the amplification of substantially the entire target. Accordingly, it is preferred that the amplification of the isolated chromosome or a part thereof is randomly primed amplification.

Amplification of the DNA from an isolated chromosome or part there of will be performed with one or more appropriate primers. As discussed above, preferably the one or more primers used will result in the random amplification of the DNA of the isolated chromosome or part thereof.

Preferably, the one or more primers used is an oligonucleotide including one or more nucleotides of random sequence. More preferably, the one or more primers is an oligonucleotide including one or more contiguous nucleotides of random sequence. More preferably, the one or more of primers is an oligonucleotide that includes six or more contiguous nucleotides of random sequence, such as a DOP primer (degenerate oligonucleotide primer). Most preferably, the one or more primers is a primer with the following nucleotide sequence:

5'-CCGACTCGAGNNNNNNATGTGG-3' (SEQ ID NO. 1);

where NNNNNN represents the degenerate sequence. "N" represents the four possible nucleotides in the DNA sequence: "A", "T", "C" and "G" for Adenine, Thymine, Cytosine and Guanine, respectively. As such, the degenerate sequence contain mixtures of various nucleotide sequences including all possible combinations of A, T, C and G at the "N" positions.

If so desired, the nucleotide sequence of the degenerate sequence can also be biased towards a particular nucleotide composition, for example GC or AT richness.

In the case of amplification using DOP primers on an isolated chromosome, or a part of a chromosome isolated by microdissection, the amplification may be performed essentially as described in Telenius *et al.* (1992) *Genomics* 18:718-725. Briefly, the amplification is performed under low stringency conditions for a low number of cycles (eg five cycles) and a second stage amplification performed under more stringent conditions for a larger number of cycles (eg 35 cycles).

Alternatively, random primed amplification may be achieved using one or more primers of fixed sequence and performing a low number of cycles of amplifcation under low stringency conditions that allow the one or more primers to prime synthesis randomly throughout the target, followed by a second stage amplification performed under more stringent conditions for a larger number of cycles.

In addition, to account for regions of small chromosomes that may present a difficulty in achieving randomly primed amplification, region specific primers can be used in conjunction with other primers that allow random amplification. For example, primers to specific regions of chromosomes 21 and 22 may be used in conjunctions with DOP primers.

Other suitable techniques for amplification of the isolated chromosome or part thereof include primer-extension pre-amplification PCR (PEP-PCR) which may be performed essentially as described in Zhang *et al.* (1992) *Proc Natl. Acad. Sci* 89:5847-5851); ligation mediated PCR, which may be performed essentially as described in Klein *et al.* (1999) *Proc. Natl. Acad. Sci.* 96:4494-4499); or alu-PCR, which may be performed essentially as in Nelson *et al.* (1989) *Proc. Natl. Acad. Sci.* 86:6686-6690).

In the case of using rolling circle amplification on a cloned genomic insert in a circular vector, rolling circle amplification may be performed using suitable conditions known in the art, such as described in Fire, A. and Xu, S-Q. (1995) *Proc. Natl. Acad. Sci* 92:4641-4645.

A number of methods known in the art can be used to deplete the amplified DNA of repetitive sequences.

Repetitive sequences are sequences present in more than one copy in the target sequence to be amplified. Repetitive sequences may be removed either prior to amplification or after amplification. For example, chromosomal DNA may be isolated and repetitive sequences removed. Alternatively, the DNA may first be amplified with appropriate primers and the repetitive DNA sequences removed from the amplified pool of nucleic acids.

Examples of repetitive sequences include simple repeated DNA (eg Alu or Kpn elements), satellite repeats, mini-satellite repeats, chromosome-specific repeats, micro-satellite repeats, repeated genes (eg rRNA genes), sequences derived from transposable elements (eg transposons with DNA or RNA intermediates), elements derived from multiple copies of viruses such as retroviruses, repeats associated with centromeres or telomeres, or repeats associated with heterochromatin.

A number of methods known in the art can be used to remove repetitive sequences For example, in many genomes, such as the human genome, a

major portion of repetitive DNA is contained in a few families of highly repeated sequences such as Alu. To remove such repetitive sequences, a blocking procedure can be used. These methods primarily exploit the fact that the hybridization rate of complementary nucleic acid strands increases as their concentration increases. Thus, if a mixture of nucleic acid fragments is denatured and incubated under conditions that permit hybridization, the sequences present at high concentration will become double-stranded more rapidly than the others. The double-stranded nucleic acid can then be removed by the direct removal of these sequences by a method known in the art.

For example, single- and double-stranded nucleic acids have different binding characteristics to hydroxyapatite. Such characteristics provide a basis commonly used for fractionating nucleic acids. The fraction of genomic DNA containing sequences with a particular degree of repetition can be obtained by denaturing genomic DNA, allowing it to reassociate under appropriate conditions, followed by separation using hydroxyapatite. Such techniques are as described in Britten *et al.* "Analysis of Repeating DNA Sequences by Reassociation" *Methods in Enzymology* 22: 363-418 (1974).

Examples of such sequences that can be used to deplete the amplified DNA of repetitive sequences include human Cot-1 DNA and Alu – repeat containing DNAs.

Alternatively, reaction with immobilized nucleic acid may be performed. For example, minimally sheared human genomic DNA is bound to diazonium cellulose or a like support. The amplified DNA, appropriately cut into fragments, is hybridized against the immobilized DNA to Cot values in the range of about 1 to 100. The material that does not bind to the immobilised nucleic acid may then be attached to the solid substrate.

In a preferred embodiment, the nucleic acid derived from the isolated chromosome or part thereof to be attached to the solid substrate is also depleted of non-chromosomal sequences, or sequences that are over-

represented due to amplification. Non-chromosomal sequences are sequences that are not normally present in the nucleotide sequence of the chromosome or part thereof, such as sequences derived from a vector or plasmid, or contaminating sequences that may be present in the originally target sample to be amplified, such as sequences derived from *E.coli*. Sequences that are over-represented due to amplification of the chromosome are sequences present after amplification of the target that have been disproportionately amplified in comparison to other sequences normally present in the target.

Non-chromosomal sequences may be depleted before or after amplification of the target sequence. In a similar manner to as described above for the depletion of repetitive sequences, non-chromosomal sequences may be depleted by using non-chromosomal sequences in excess in a hybridisation reaction with the target or the amplified DNA, or by attaching the non-chromosomal sequences to a solid support and using theses sequences to deplete the DNA of these sequences.

In the case of depleting over-represented sequences due to amplification, the target sequences may be depleted from the target before amplification by similar methods described above, or the over-represented amplified sequences may be depleted after amplification. As will be appreciated, identification of the actual sequence being over-represented is necessary, and will depend on the primers being used and the nature of the target to be amplified.

In a preferred form, the present invention also provides a nucleic acid attached to a solid substrate, wherein the nucleic acid is the product of randomly primed amplification of an isolated chromosome or part thereof and the nucleic acid is depleted of one or more of repetitive sequences, non-chromosomal sequences or sequences that are over-represented due to amplification of the chromosome or part thereof.

The nucleic acid from the isolated chromosome or part thereof may also subjected to size selection before attachment to the solid substrate. Preferably,

the amplified DNA attached to the solid substrate is less than 10 kb in size. More preferably, the amplified DNA attached to the solid substrate is less than 3 kb in size.

Size selection may be performed by a suitable method known in the art. For example, the amplified DNA may be electrophoresed on an agarose gel, and DNAs with a size in the range from 150 to 3000 bp may be isolated.

In a preferred form, the nucleic acid attached to the solid substrate is the product of randomly primed amplification of an isolated chromosome or part thereof, wherein the nucleic acid has been size selected. In this case, the randomly amplified DNA attached to the solid substrate is preferably less than 10 kb in size. More preferably, the randomly amplified DNA attached to the solid substrate is less than 3 kb in size. For example, the randomly amplified DNA may be electrophoresed on an agarose gel, and DNAs with a size in the range from 150 to 3000 bp may be isolated.

The nucleic DNA may be attached to a solid substrate by a suitable method known in the art, including passive adsorption or covalent linkage. For example, amplified DNA may be attached to a glass substrate by passive adsorption by spotting samples onto a Polysine™ microscope glass slide (Menzel-Glaser, Gemany) and processing of the slide by dehydration, snap-drying, fixation through UV cross linking, and chemical blocking by using succinic anhydride. In the case of covalent linkage, the nucleic acid may be attached to the solid substrate by a suitable method known in the art.

It is preferred that more than one nucleic acid is attached to the solid substrate, to produce an array of deposited nucleic acids. Such an array can be manufactured in any desired manner known in the art, including robotic deposition of the nucleic acids. Examples of methods for producing arrays are essentially as described in U.S. Patents 5,486,452, 5,830,645, 5,807,552, 5,800,992 and 5,445,934.

Accordingly, in a preferred form, the present invention also provides an array of nucleic acids attached to a solid substrate, wherein each nucleic acid in the array is derived from an isolated chromosome or part thereof and each nucleic acid is depleted of repetitive sequences.

In another preferred form, the present invention also provides an array of nucleic acids attached to a solid substrate, wherein each nucleic acid in the array is the product of randomly primed amplification of an isolated chromosome or part thereof and each nucleic acid is depleted of one or more of repetitive sequences, non-chromosomal sequences or sequences that are over-represented due to amplification of the chromosome or part thereof.

Any suitable amount of nucleic acid may be deposited on the solid substrate. The amount of nucleic acid deposited can be from about 0.05 nl to about 5.0 nl of a nucleic acid solution of 0.15 - 1 μg/μl nucleic acid concentration. For example, for a density of 1,000 DNAs deposited/cm, the individual amount deposited is about 0.2 nl to about 2.0 nl of 1 μg/μl solution. The DNA is provided in any solvent that will permit deposition of the nucleic acid.

The array having deposited nucleic acids may be produced in any arrangement. For example, the nucleic acids can be located in one portion of the array or can be interspersed among other deposited nucleic acids. The regularity of a two dimensional array is preferred.

It is also preferred that the array include various control nucleic acids, such as, for example, spotted nucleic acids of known copy number for a particular expressed gene or genomic sequence. For example, genomic DNA extracted from cell lines with 1 or more copies of a particular chromosome can be used, or the entire DOP-PCR products of amplification of DNA from a single cell can also be used.

In another form, the present invention also provides a kit for comparing at least one chromosome or part thereof from a cell with a first karyotype with the

corresponding chromosome or part thereof from a cell with a second karyotype, the kit including a nucleic acid attached to a solid substrate, wherein the nucleic acid is derived from an isolated chromosome or part thereof and the nucleic acid is depleted of repetitive sequences.

In a further form, the present invention also provides a kit for comparing at least one chromosome or part thereof from a cell with a first karyotype with the corresponding chromosome or part thereof from a cell with a second karyotype, the kit including a nucleic acid attached to a solid substrate, wherein the nucleic acid is the product of randomly primed amplification of an isolated chromosome or part thereof and the nucleic acid is depleted of one or more of repetitive sequences, non-chromosomal sequences or sequences that are over-represented due to amplification of the chromosome or part thereof.

In another form, the present invention also provides a kit for comparing at least one chromosome or part thereof from a cell with a first karyotype with the corresponding chromosome or part thereof from a cell with a second karyotype, the kit including an array of nucleic acids attached to a solid substrate, wherein each nucleic acid in the array is derived from an isolated chromosome or part thereof and each nucleic acid is depleted of repetitive sequences.

In another form, the present invention also provides a kit for comparing at least one chromosome or part thereof from a cell with a first karyotype with the corresponding chromosome or part thereof from a cell with a second karyotype, the kit including an array of nucleic acids attached to a solid substrate, wherein each nucleic acid in the array is the product of randomly primed amplification of an isolated chromosome or part thereof and each nucleic acid is depleted of one or more of repetitive sequences, non-chromosomal sequences or sequences that are over-represented due to amplification of the chromosome or part thereof.

The various kits of the present invention are also suitable for detecting a chromosomal abnormality in a cell.

SEQUENCE LISTING

<210> SEQ ID NO. 1
<211> 21
<212> DNA
<213> Artificial Sequence
<221> misc_feature
<222> (10)..(15)
<223> n is a, c, g, or t
<400> SEQ ID No. 1
ccgactcggn nnnnnatgtg g

Description of the Preferred Embodiments

Reference will now be made to experiments that embody the above general principles of the present invention. However, it is to be understood that the following description is not to limit the generality of the above description.

Example 1

Human chromosome-specific DNA libraries

A complete set of repeat-depleted, PCR-amplifiable, human chromosome-specific painting probes was kindly provided by Drs. A.Bolzer and M.R. Speicher (Institut für Anthropologie and Humangenetik, LMU München, München, Germany).

Because of their ability to uniformly paint the whole target chromosome (arms), these probes were selected in this project as DNA libraries of human chromosomes. The DNA libraries were generated by microdissection for 15 chromosomes of No.1, 3, 6, 7, 9, 12, 13, 14, 15, 17, 19, 20, 21, 22, and X) or flow-sorting for 9 chromosomes of No. 2, 4, 5, 8, 10, 11, 16, 18, and Y. In order to avoid cross-hybridisation among the p arms of five acrocentric chromosomes (13~15, 21, and 22), only q arms of these chromosomes were microdissected into their corresponding DNA libraries (Guan *et al.* (1994) *Genomics* 22:101-107).

Using subtracters including human Cot-1 DNA, chromosome centromere-specific probes, and aphoid region-specific probes, further depletion of repetitive sequences out of these probes was successfully conducted by affinity chromatography. Briefly, repeat sequences were labelled with biotin and allowed to hybridise to the library containing repeat sequences. After hybridisation streptavidin-magnetic bead affinity chromotography was used to remove the repeat sequences bound to the biotin labelled repeat sequences.

Without addition of human Cot-1 DNA to suppress repetitive sequences, these repeat-depleted DNA libraries achieve high specific FISH signals on their corresponding target chromosomes (q arms) as described in Craig *et al.* (1997) *Hum. Genet.* 100:472-474 and Bolzer *et al.* (1999) *Cytogenet. Cell Genet.* 84:233-240.

Example 2

Preparation of human chromosome-specific DNA libraries

Successful re-amplification of the human DNA library described in Example 1 was achieved by single round of degenerate oligonucleotide-primed PCR (DOP-PCR) for 30-35 cycles of high-stringent cycling conditions, essentially as described in Telenius *et al.* (1992) Genomics 13:718-725.

Briefly, amplification was carried out in a Minicycler (MJ Research, USA) in a volume of 50 µl, which contained about 50 ~100 ng of source probes, Taq DNA ploymerase buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], Perkin Elmer, USA), 2.0 µM primer 6MW(5'-CCGACTCGAGNNNNNNATGTGG-3'), 2.5 mM $MgCl_2$, 0.25mM of each dNTP, and 5 U Taq DNA polymerase (Perkin Elmer, USA). After an initial denaturation step of 95°C for 4 min, 30-35 cycles were followed using cycling conditions of 94°C for 1 min, 62°C for 1 min, and 72°C for 3 min with an addition of 10 seconds per cycle to the extension time. Finally an extension step of 72°C for 10 min was added at the end of cycling amplification.

5 µl of PCR products was electrophoresed on 1% agarose gels in 0.5 X TBE (Tris, borate, EDTA) prestained with ethidium bromide and photographed. The results are shown in Figure 1.

As can be seen, successful amplification of the whole set of repeat-depleted human chromosome-specific DNA libraries was achieved by one round of DOP-PCR amplification of 30-35 cycles by using the high stringent cycling conditions of the traditional DOP-PCR. All PCR products of reamplified DNA libraries were

smears with the majority less than 1 kb after 30 min run on 1% Agarose gels. However, differences of smears were obvious. Wider smears extending up to more than 3 kb were seen for products of eleven different chromosomes (No.1, 2, 4, 5, 8, 9, 10, 11, 16, 21, and Y), only three of which were microdissection-derived probes (No.1, 9, and 21).

Comprehensively optimising the cycling conditions of DOP-PCR, which included both temperature and duration for both steps of annealing and elongation, the numbers of amplification cycles, initial DNA quantities of templates, and salt concentrations of $MgCl_2$, failed to remove the differences in sizes of the PCR products.

Purification of the PCR products was then conducted by Ultrapure PCR purification kits(#12500-250, Mo Bio Laboratories, Inc., CA, USA). Purified products were either used immediately or stored at -20°C for one year without any visible loss of their specificities, as determined by FISH signals.

Example 3

Fluorescence in situ hybridisation using re-amplified DNA libraries

FISH experiments were carried out to confirm the specificities for the DNA libraries of human chromosomes reamplified by using metaphase chromosomes of peripheral lymphocytes from a normal human male.

Fluorescence in situ hybridisation was carried out as follows:
SpectrumGreen-dUTP or SpectrumRed-dUTP (Vysis, USA) was used to label the amplified DNA libraries. Labelling reaction was carried out in a Minicycler (MJ Research, USA) with a volume of 50 µl using similar DOP-PCR cycling conditions used above to reamplify source probes of DNA libraries (Example 2). Low concentration of dNTP was the only exception employed here with 0.16 mM for each of dGTP, dCTP, and dATP, and 0.12mM for dTTP with addition of 0.04mM for either SpectrumGreen-dUTP or SpectrumRed-dUTP. Purification of

PCR products was conducted by Ultrapure PCR purification kits(#12500-250, Mo Bio Laboratories, Inc., CA, USA). For FISH experiments, 1μg of purified labelled products was mixed with 20 μg of human Cot-1 DNA(GIBICO, BRL) and 50μg of Salmon sperm DNA (GIBICO, BRL). The probe mixture was precipitated with ethanol and resuspended in 10 μl of hybridiztaion solution, which was consisted of 50% deionized formamide, 3 X SSC, 0.1% SDS, 10% dextran sulfate, and 5 X Denhardt's solution.

After denaturation at 80°C for 10 min and preannealing at 37°C for 30 min, probes were hybridized to denatured metaphase chromosome spreads at 37°C overnight. After hybridization, the slides were washed twice with 2 X SSC at 60°C for 10 min and then twice with 0.1 X SSC at 60°C for 5 min. After that, slides were further washed once in 0.1 X SSC at room temperature for 5 min and then briefly rinsed in H_2O for a few seconds. After being dried by air in the dark, slides were counterstained with 40 μl of DAPI solution containing antifading medium and covered with coverslips sealed by nailsticks. FISH signals were obtained and photographed by microscope of AHBT3(Olympus, Tokyo, Japan) using (1) excitation with blue for Green signals and (2) excitation Triple for Red signals.

As shown in Figure 2, except for very faint signals for the DNA library of chromosome 21, all the others uniformly painted their whole target chromosomes or q arms.

Example 4

Manufacture of DNA arrays

(i) DNA arrays of PCR amplified libraries of human chromosomal DNA (First Generation Array)

DNA arrays were made using the Microarray facilities of the University of Adelaide, South Australia.

Briefly, all re-amplified DNA libraries of human chromosomes were resuspened in spotting buffer of 3 X SSC with final DNA concentration of around 100ng/μl, and then 8μl of each suspension solutions were loaded into the wells of a 384-well plate. Subsequently, a microarrayer sampled wells from this plate and spotted 8 replicates for every DNA sample on Polysine™ microscope glass slides (Menzel-Glaser, Gemany). Post-processing of the printed array slides included dehydration, snapping-dry, fixation through UV cross linking, and chemical blocking by using succinic anhydride. Finally, after being dried by centrifuging at 500 rpm for 5 min, array slides could be used immediately or stored in slide box for a short period of time in the dark.

Thirty array slides were manufactured. As shown in Figure 3, two replicate arrays were spotted on every slide. Each array had 4 blocks. Within each array four blocks were referred from left to right as the 1st, 2nd, 3rd, and 4th block, which had 7, 7, 6, and 6 columns, respectively. Each column was consisted of 4 replicates of one DNA library of a single human chromosome. From left to right, their column's orders corresponding to DNA libraries of human chromosomes were No.1-5-9-3-17-21-negative, No.2-6-10-14-18-22-positive, No.3-7-11-15-19-X-blank, and No.4-8-12-16-20-Y-blank for the 1st, 2nd, 3rd, and 4th block, respectively.

(ii) DNA arrays of PCR amplified libraries of human chromosomal DNA after size selection (Second Generation Array)

A DNA array with size selected products was produced by electrophoresis of the re-amplified DNA libraries described in Example 2 on a 1% Agarose gel. After staining, DNAs in the size range of 150 – 3000 bp were excised from the gel and isolated, as shown in Figure 4.

The resulting size selected DNAs were then spotted onto an array along with the original DNA libraries prior to size selecion as described previously, with the following changes: DNA was resuspended in 150 mM sodium phosphate, pH

8.0 at an approximate concentration of 170ng/ µl. An amount of approximately 0.6nl was spotted for each spot using Stealth Micro Spotting Pins (Catalogue SMP3) from TeleChem. The slides used were SuperAmine slides from TeleChem (Sunnyvale, CA) which were used straight from the box. The Arraying facility which performed the microarraying was the Clive & Vera Ramaciotti Centre for Gene Function Analysis microarray facility located at the University of New South Wales which uses a ChipWriter Pro (BioRad) microarrayer.

Example 5

Isolation and preparation of single cells

Single lymphocytes isolated from normal male or normal female peripheral blood were used as reference cells, and single amniocytes from amniocyte cell cultures of pregnancies of trisomies 13 and 18 were used as test cells. Cytogenetic analysis was used to confirm the normal karyotypes of the 46, XX and 46, XY reference samples. Using an inverted light microscope and finely pulled glass pipettes, single cells were selected and transferred into 0.5 ml PCR tubes essentially as described in Hussey *et al.* (1999) *Mol. Hum. Reprod.* 5:1089-1094, which could be used immediately or frozen for a period of time.

Example 6

First round DOP-PCR for random amplification

Lysis of single cells was achieved by 5µl of lysis buffer (200mM KOH, 50mM dithiothreitol) for 10 min at 65°C followed by neutralization with 5µl of neutralization buffer (300mM KCl, 900mM Tris-HCl, Ph 8.3, 200mM HCl). To the 10 µl of lysed and neutralized solution of single cells was added: 5µl of K^+-free PCR buffer (100mM Tris-HCl, pH 8.3, gelatin 1mg/ml), 5 µl of 25 mM $MgCl_2$, 4µl of 2.5mM of each dNTP, 5µl of 20µM of DOP-PCR 6MW, 5U of Taq polymerase (Perkin Elmer, Norwalk, CT, USA), and ultrapure water (Biotech

International, Perth, WA, Australia) to a volume of 50 µl. These PCR tubes were placed into a MJ Research Minicycler (Boston, MA, USA) for an initial denaturation step of 95°C for 5 min. The subsequent cycling conditions contained 8 cycles of low stringent amplification of 94°C for 1 min, 30°C for 1.5 min, 72°C for 3 min with a ramp of 1°C per 4 seconds for increasing temperature from 30°C to 72°C, and 26 cycles of high stringent amplification of 94°C for 1 min, 62°C for 1 min, 72°C form 3 min with an addition of 14 seconds per cycle to the extension step. Finally an extension step of 72°C for 10 min was added at the end of cycling amplification. PCR products were ready for seeding the second round DOP-PCR for labelling.

Example 7

Second round DOP-PCR for Cy3/Cy5 labelling

5 µl of first round DOP-PCR products (1/10 vol) was transferred into fresh 0.5 ml PCR tubes and subjected to a second round DOP-PCR amplification for labelling of Cy3-dUTP/Cy5-dUTP (PA 53022/PA 55022, Amersham Phamacia Biotech, USA). Amplification was carried out in a volume of 50 µl for 25 cycles using a MJ Research Mimicycler (Boston, WA, USA), and the similar cycling conditions were applied as labelling DNA libraries for FISH (described above) with an exception of replacing SpectrumRed or SpectrumGreen with either Cy3-dUTP or Cy5-dUTP. Either Cy3-dUTP or Cy5-dUTP was used at a concentration of 0.04mM. PCR products were then purified by Ultraclean™ PCR clean-up kits(#12500-250, Mo Bio Laboratories, Inc., CA, USA), and eluted in 50 µl of either 10 mM Tris-HCl or H_2O. 5 µl of purified PCR products was electrophoresed on 1% agarose gels in 0.5 X TBE (Tris, borate, EDTA) prestained with ethidium bromide and photographed. The remaining could be either immediately used in microarray/CGH experiments or stored at -20°C for a short period of a few weeks.

Example 8

Microarray/CGH analysis

Equal amounts (5 ~ 10 µl) of Cy3-labeled test and Cy5-labelled reference single-cell DOP-PCR products were mixed with 70µg of human Cot-1 DNA (GIBICO, BRL) and 20µg of Salmon sperm DNA (GIBICO, BRL). The resultant DNA mixture was precipitated with ethanol and resuspended in 10 µl of hybridiztaion solution containg 50% deionized formamide, 2 X SSC, 0.1% SDS, 10% dextran sulfate, and 5 X Denhardt's solution. The hybridization mixture was heated to 80°C for 10 min to denature DNA probes followed by preannealing of repetitive sequences at 37°C for 180 min. Hybridization was carried out at 37°C for 17~20 hrs. Post-hybridization washing included three times of 50% formamide/2 X SSC, pH 7.0 at 45°C for 10 min, twice of 2 X SSC at 45°C for 5 min , and once of 1 X SSC at room temperature for 10 min. Finally, slides were briefly rinsed in H_2O for a few seconds and dried by air in the dark, and then scanned as soon as possible.

Example 9

Slides scanning and data analysis

Microarray slides were scanned by a dual laser scanner called GenePix 4000B (Axon Instruments, Inc., CA, USA), which is capable of scanning Cy3 (at 532nm) and Cy5 (at 635nm) simultaneously and produces a ratio image in real time. These images were further analysed by a software of GenePix Pro 3.0.6.66 (Axon Instruments, Inc. USA). This software calculated both signal and local background intensities at both wavelengths (Cy3/Cy5) for all DNA dots and produced numerous raw data, among which five different ratios of Ratio of Medians, Ratio of Means, Median of Ratios, Mean of Ratio and Regression Ratio were most important. This software also presented a normalization factor for each of the five ratios by using global normalization on the assumption that the mean value of all the analysed features is 1.0. The normalized ratios could

be either combined or compared across different array experiments. Ratios of Medians were selected in this study, and the averages of normalized ratios (Cy5/Cy3) from all suitable dots of the same probes were finally used for final analyses. A normalized ratio value of 1.0 was considered that there was no difference of copy numbers between the test and reference. Large changes in ratios indicated significant differences of copy numbers. A cut off threshold of less than 0.80 (autosomal) or 0.75 (sex chromosome) for trisomies and greater than 1.2 (autosomal) or 1.25 (sex chromosome) for monosomies was used in this study to determine aneuploidies of single copy changes. These criteria of threshold cutoffs is frequently used in comparative genomic hybridization for diagnosing single copy changes of genomic sequences.

The definition of Ratio of Medians was the ratio of the median intensities of each feature (DNA dot) for each Wavelength, with the median background subtracted. The steps used to determine the Normalization Factor for the Ratio of Medians by the software were as follows:

1) The Log value for each feature's Ratios of Medians Value is determined
2) The Average of all of the Log values is calculated ("Avglog")
3) The True average is calculated ("TrueAvg"). TrueAvg=10^Avglog
4) The Normalization Factor (NF) is determined. NF=1/TrueAvg
5) The Normalizated Ratios of Medians were calculated by NF X Ratios of Medians

Example 10

Results of single-cell DNA microarray/CGH

Single amniocytes of 47, XX, +13 and 47, XY, +18 were used as test samples and labelled with Cy3-dUTP (green), whereas single lymphocytes from a normal male 46, XY was used as a reference sample and labelled by Cy5-dUTP (red). After post-hybridization washing, array slides were dried and scanned immediately by GenePix 4000B, which could produced both single-wavelength

and double-wavelength images. These images could be saved in 24-bit JPEG images and by default as 16-bit unsigned TIFF images. The entire CGH procedure took approximately 30 hours.

Figure 5 was obtained from single-cell microarray/CGH experiments of 47, XX, +13 versus 46, XY. Preliminary analysis showed several chromosomal regions that appeared greener or redder on the target DNA dots. However, the final interpretation of copy numbers for target chromosomes was obtained from ratios of their corresponding DNA dots (see graphs).

Similarly, Figure 6 was obtained from single-cell microarray/CGH experiments of 47, XY, +18 versus 46, XY. The graph gives the final interpretation of copy numbers for target chromosomes obtained from ratios of their corresponding DNA dots.

16-bit unsigned TIFF images are standard uncompressed graphic file formats that can be read by many graphics and imaging programs. These images were used for extraction of data. The TIFF images were analysed in this study by GenePix Pro 3.0.6.66, which produced comprehensive data report sheet (in Excel format) for every microarray/CGH experiments. Ratios of Medians were selected in this study to interpret the final results, and in this study averaged ratios of medians from all suitable DNA dots of same DNA probes were finally used. Averaged ratios of medians of all human chromosomes for the images of Figures 5 and 6 are given in Table 1.

Table 1

Name of Chromosome	Averages of ratios 47, XX, +13 v 46, XY (Cy3/Cy5)	Averages of ratios 47, XY, +18 v 46, XY (Cy3/Cy5)
No. 1	1.076990539	1.067886663
No. 2	1.05202167	1.162437071
No. 3	1.048407129	0.962005996
No. 4	1.172823435	1.171398634
No. 5	0.980301567	1.466064292
No. 6	0.888891728	0.971954515
No. 7	0.92265915	0.95221539
No. 8	0.926083452	0.957544954
No. 9	0.933597893	1.25382922
No. 10	0.846801349	1.29962399
No. 11	1.213487021	1.180399675
No. 12	0.853697513	0.859994198
No. 13	0.647050399	1.10381187
No. 14	0.853412154	0.876101324
No. 15	0.908581464	0.850519418
No. 16	1.015400663	1.12276143
No.17	0.765236378	0.723399453
No. 18	0.9321711	0.669077381
No. 19	0.991382989	0.871995586
No. 20	0.773321535	0.831411945
No. 21	1.28601564	1.074005791
No. 22	0.917237339	0.872627238
X	0.732895748	1.425362216
Y	1.568758354	0.66007634

A graphical representation of the results in Table 1 is in Figure 6.

The data in Table 1 show that in the single-cell microarray/CGH experiments averaged ratios of medians for dots of 13, 18, X, and Y were 0.6470, 0.9321, 0.7328, and 1.568, respectively for 47, XX, +13 versus 46, XY.

In the case of 47, XY, +18 versus 46, XX, these ratios were shifted to 1.1038, 0.6691, 1.4254, and 0.6601 for chromosomes 13, 18, X and Y, respectively. If cut-off threshold of 0.75-1.25 applied to determine single-copy changes of chromosomes, trisomies 13 and 18 plus the differences of copy numbers of chromosome X and Y could be correctly established for all testing and reference samples used in these two single-cell microarray/CGH experiments.

The expected averaged ratios of medians for all other 20 different chromosomes should be within the cut-off threshold of 0.75-1.25, as there were no any differences of copy numbers for these chromosomes between the test and reference samples. The data in Table 1 demonstrate that the majority of the other 20 chromosomes fit quite well within this threshold. Averaged ratios of medians were found to be 1.2860 for chromosome 21 for 47, XX, +13 versus 46, XY, and 1.47660, 1.2996, and 0.72339 for chromosomes 5, 10 and 18 for 47, XY, +18 versus 46, XX.

Example 11

Reproducibility of CGH performed with DNA array with size selected PCR amplified DNA (Second Generation Array).

Experimental protocols were the same as described above with the exception that the second generation array with size selected DNAs was used. This array also contains the original DNA libraries prior to size selection. Aliquots of normal male and normal female cell DOP-PCR reactions were labelled with either Cy3 or Cy5 and hybridised as per the combinations described in Table 2 below.

Table 2

Slides	Microarray/CGH experiments	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y (37)	Y (26)
NH1 rh	DH_1Cy_5/NH_5Cy_3		#							#	#															
NH3 rh	$DH_{25}Cy_5/NH_{42}Cy_3$			#																					#	#
NH6 lh	DH_1Cy_5/NH_5Cy_3																								#	
NH6 rh	$DH_1Cy_5/NH_{13}Cy_3$								#																	
NH7 lh	$DH_1Cy_5/NH_{13}Cy_3$																						#			#
NH7 rh	$DH_{13}Cy_5/NH_{25}Cy_3$																									#
NH8 lh	$DH_{13}Cy_5/NH_{13}Cy_3$																									#
NH8 rh	$DH_{13}Cy_5/NH_{13}Cy_3$											#							#		#		#			#
NH9 lh	DH_1Cy_5/DH_1Cy_3																									
NH10 lh	$DH_1Cy_5/NH_{13}Cy_3$																									#
NH10 rh	$DH_{13}Cy_5/NH_{13}Cy_3$																								#	#
NH11 rh	$DH_{13}Cy_5/NH_{25}Cy_3$																							#	#	#
NH12 lh	NH_5Cy5/NH_5Cy3																									
Nh12 rh	$DH_{13}Cy_5/NH_{25}Cy_3$				#	#						#													#	#
Total Expected ratios	14 separate microarray/CGH experiments	14	13	14	12	13	14	14	13	13	13	12	14	14	14	14	14	13	14	14	13	14	12	13	9	9

(1) Total single lymphocytes used here including 10 DH cells and 11 NH cells
(2) Cells used more than once including DH1, NH9, DH13

Table 2 shows that single cell comparative genomic hybridisation experiments using our array are extremely reproducible with all chromosomes (except the Y chromosome) producing perfect results in at least 13 of the 14 experiments. The size selected Y chromosome spots were more accurate than the non-size selected ones. The data corresponding to one of the above experiments (NH7 lh) is shown in Figure 7. Table 3 shows the ratios calculated by the computer:

Table 3

NH7 Ih - Normalization of Median of ratios					
	Raw Median of ratios	Log Value	NF	Normalization Median of Ratios	Log2 value of normalized median of ratios
1	0.575125	-0.240238	1.826851	1.050667624	0.071306348
2	0.463125	-0.334302	1.826851	0.846060323	-0.241167565
3	0.619875	-0.207696	1.826851	1.132419202	0.179408117
4	0.53225	-0.273884	1.826851	0.972341392	-0.040465158
5	0.49625	-0.304299	1.826851	0.906574759	-0.141502101
6	0.592625	-0.22722	1.826851	1.082637515	0.114550285
7	0.60325	-0.219503	1.826851	1.102047805	0.140186808
8	0.502125	-0.299188	1.826851	0.917307508	-0.124522647
9	0.459125	-0.338069	1.826851	0.838752919	-0.253682212
10	0.501375	-0.299837	1.826851	0.91593737	-0.126679142
11	0.642125	-0.19238	1.826851	1.173066634	0.230284966
12	0.55375	-0.256686	1.826851	1.011618686	0.016665591
13	0.548875	-0.260527	1.826851	1.002712788	0.003908426
14	0.558875	-0.252685	1.826851	1.020981297	0.029956438
15	0.543875	-0.264501	1.826851	0.993578533	-0.009294091
16	0.619625	-0.207871	1.826851	1.131962489	0.178826151
17	0.58675	-0.231547	1.826851	1.071904766	0.100176734
18	0.543125	-0.2651	1.826851	0.992208395	-0.011284931
19	0.5925	-0.227312	1.826851	1.082409158	0.114245951
20	0.5265	-0.278602	1.826851	0.961836999	-0.056135672
21	0.46775	-0.329986	1.826851	0.854509508	-0.226831549
22	0.5675	-0.246034	1.826851	1.036737886	0.052051189
X	0.412375		1.826851	0.75334764	-0.40861233
Y	0.49825		1.826851	0.910228461	-0.135699398

Example 12

Single cell CGH using DNA array with size selected PCR amplified DNA libraries (Second Generation Array) for the detection of trisomy 18 and gender.

Single amniocytes of 47, XY, +18 were used as the test sample against single a single lymphocte 46, XX using the second generation array as described previously. Figure 8 shows the results of the hybridisation to the array. The raw data and normalised ratios is shown in Table 4.

Table 4

NH25 Ih - Normalization of Median of ratios					
	Raw Median of ratios	Log Value	NF	Normalization Median of Ratios	Log2 value of normalized median of ratios
1	0.646375	-0.189515449	1.6053857	1.037681182	0.053363257
2	0.592875	-0.227036862	1.6053857	0.951793047	-0.07128018
3	0.6595	-0.1807852	1.6053857	1.058751869	0.082364517
4	0.603879	-0.219050073	1.6053857	0.969458711	-0.044748639
5	0.592625	-0.227220032	1.6053857	0.9513917	-0.071888655
6	0.6905	-0.160836317	1.6053857	1.108518826	0.148633272
7	0.7086667	-0.149557974	1.6053857	1.137683386	0.186099116
8	0.629	-0.201349355	1.6053857	1.009787605	0.014051874
9	0.58425	-0.233401279	1.6053857	0.937946595	-0.092422314
10	0.646625	-0.189347509	1.6053857	1.038082528	0.053921143
11	0.62125	-0.206733598	1.6053857	0.997345866	-0.003834196
12	0.62325	-0.205337713	1.6053857	1.000556638	0.000802835
13	0.62875	-0.201522002	1.6053857	1.009386259	0.013478352
14	0.63014286	-0.20056098	1.6053857	1.011622336	0.016670797
15	0.59485714	-0.225587321	1.6053857	0.954975146	-0.066464908
16	0.656	-0.183096161	1.6053857	1.053133019	0.074687672
17	0.67825	-0.168610198	1.6053857	1.088852851	0.122809
18	0.48228571	-0.316695606	1.6053857	0.774254582	-0.369120079
19	0.5755	-0.239954672	1.6053857	0.92389947	-0.114192215
20	0.663	-0.178486472	1.6053857	1.064370719	0.090000727
21	0.5695	-0.244506272	1.6053857	0.914267156	-0.129312301
22	0.67057143	-0.173554954	1.6053857	1.076525785	0.106382875
X	0.69885714		1.6053857	1.121935259	0.165989428
Y	0.69525		1.6053857	1.116144408	0.158523697

Finally, it will be appreciated that various modifications and variations of the methods and compositions of the invention described herein will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in the fields of the detection of chromosome abnormalities, prenatal diagnosis and preimplantation genetic diagnosis,

molecular biology or related fields are intended to be within the scope of the present invention.

DATED: 2 April 2003

PHILLIPS ORMONDE & FITZPATRICK

Attorneys for:

The University of Adelaide

Figure 1

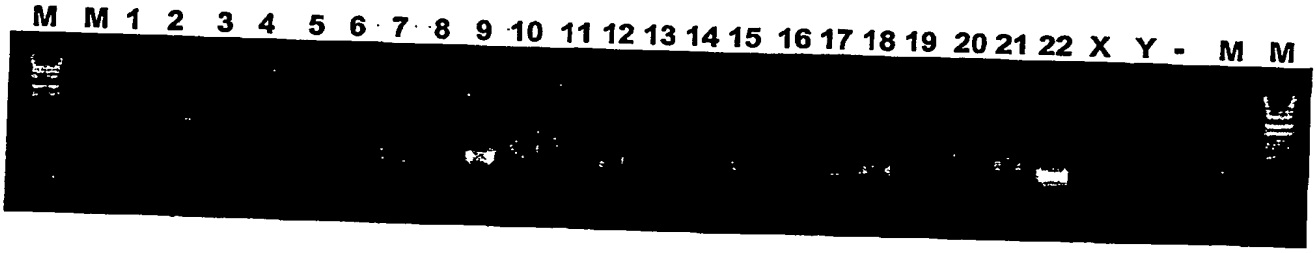


Figure 2

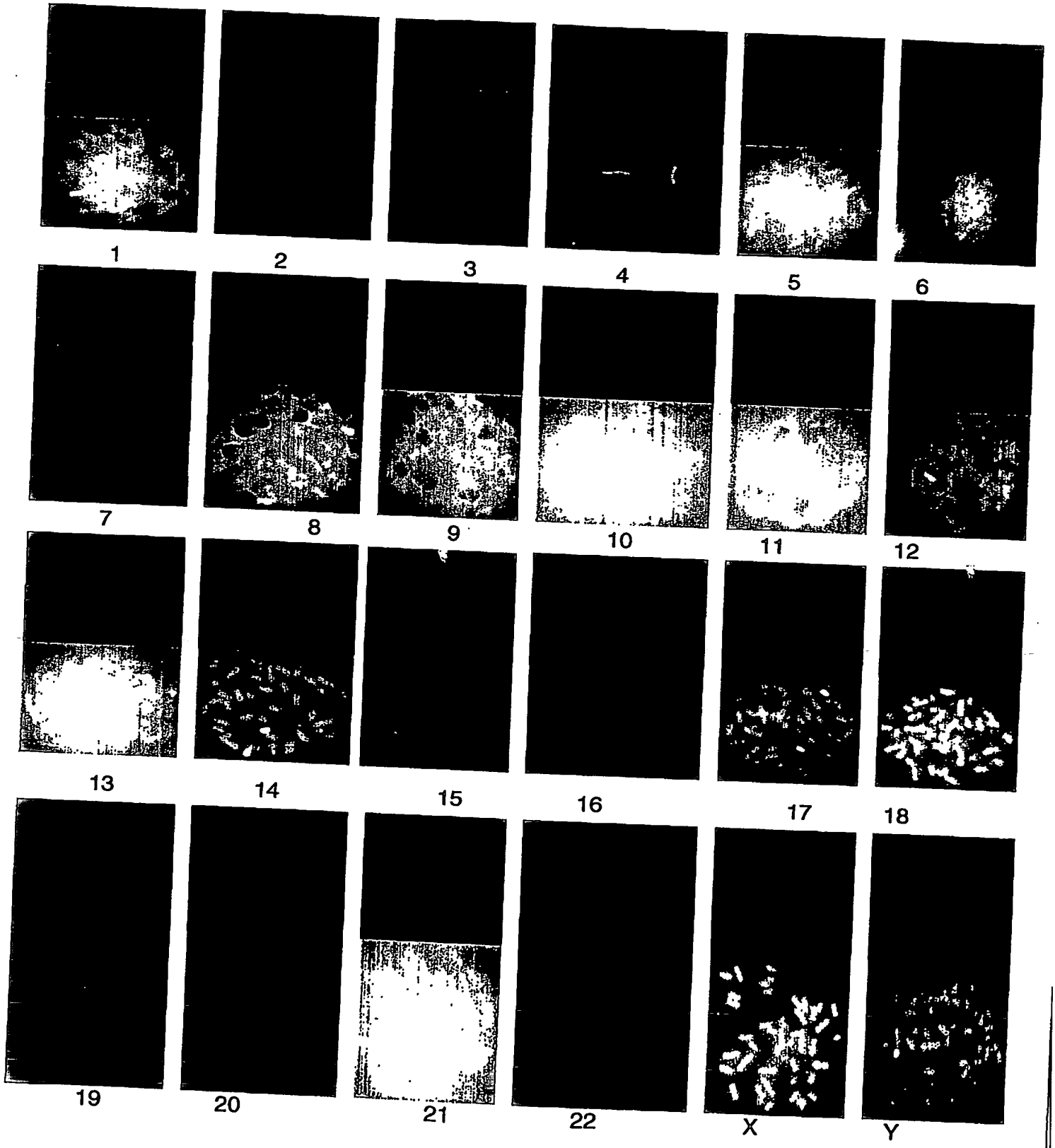


Figure 3

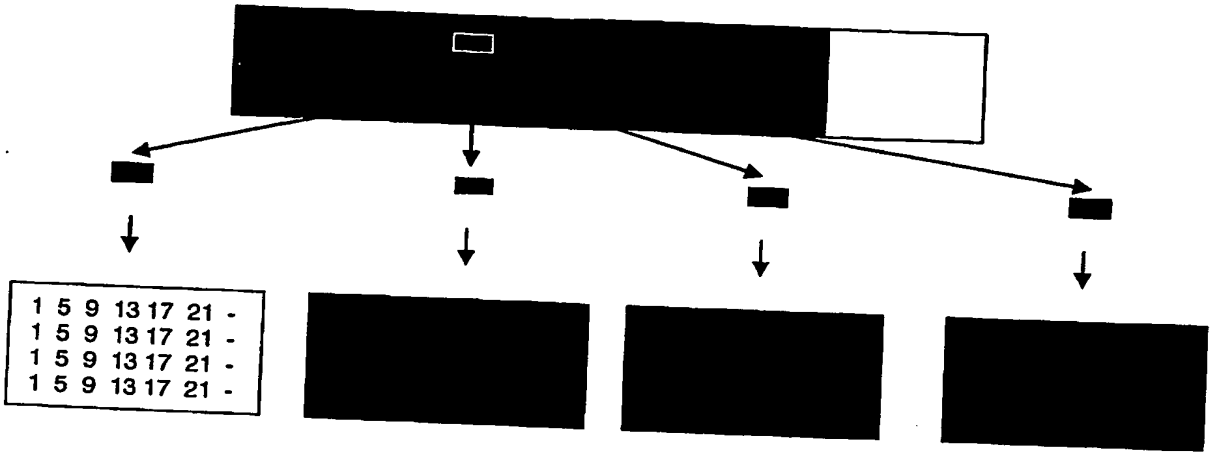


Figure 4

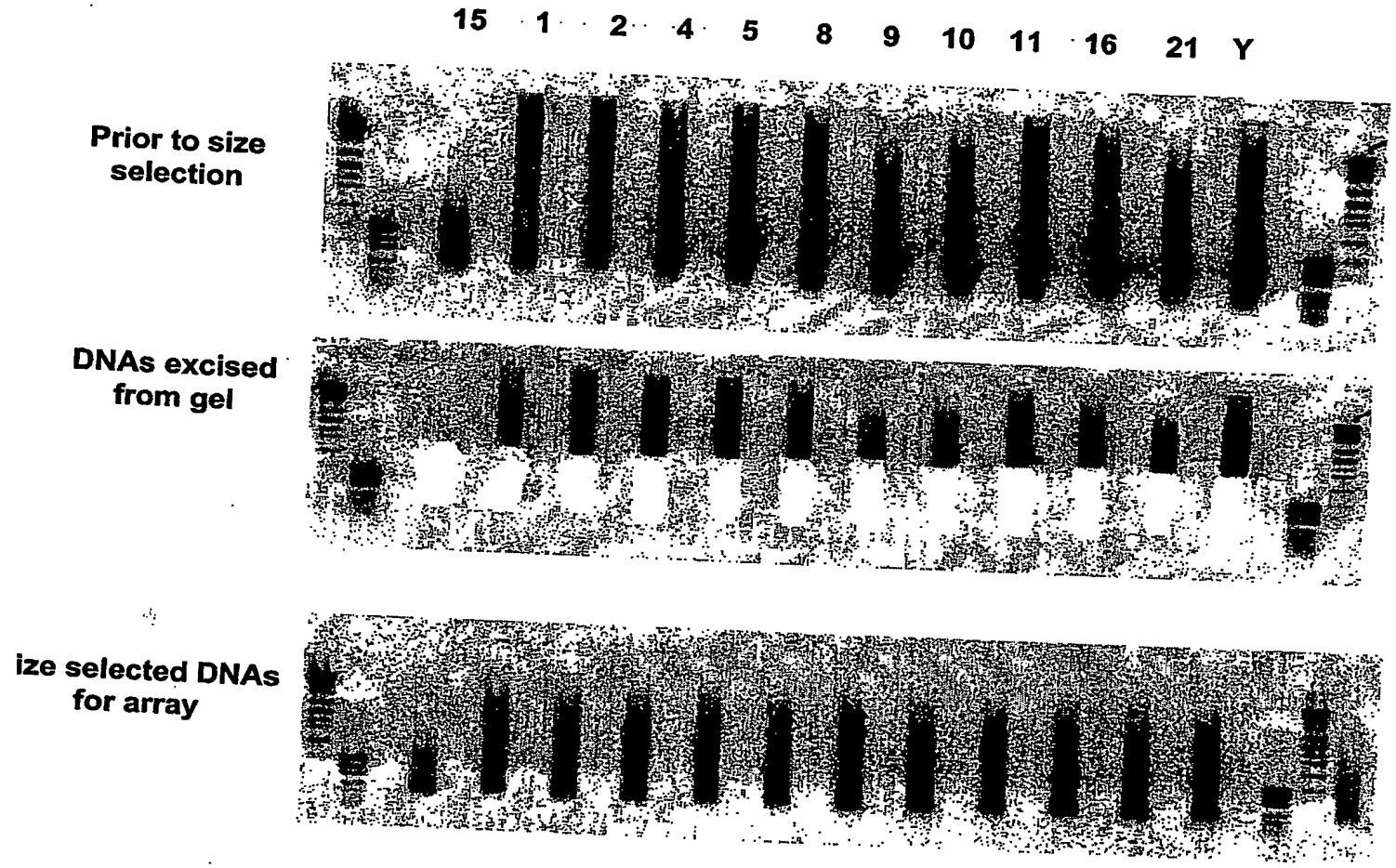
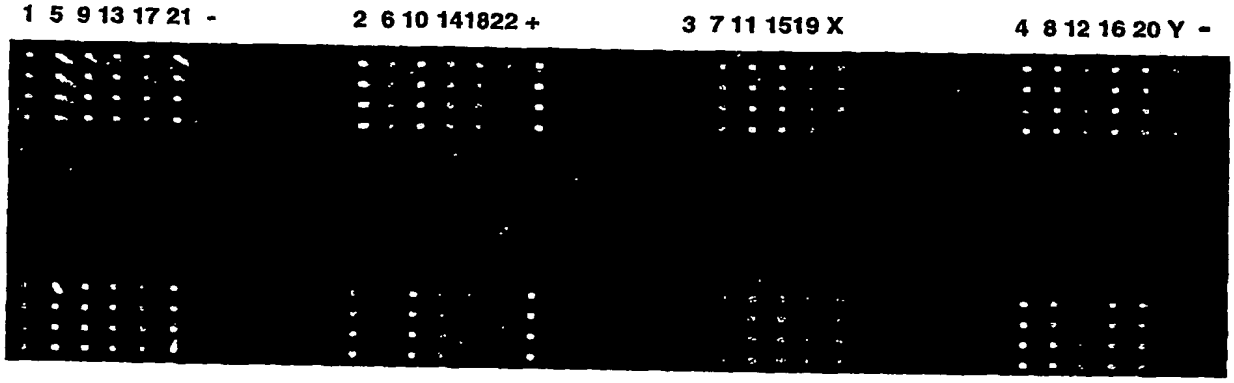

15 1 2 4 5 8 9 10 11 16 21 Y
Prior to size selection
DNAs excised from gel
ize selected DNAs for array

Figure 5


1 5 9 13 17 21 -
2 6 10 141822 +
3 7 11 1519 X
4 8 12 16 20 Y -

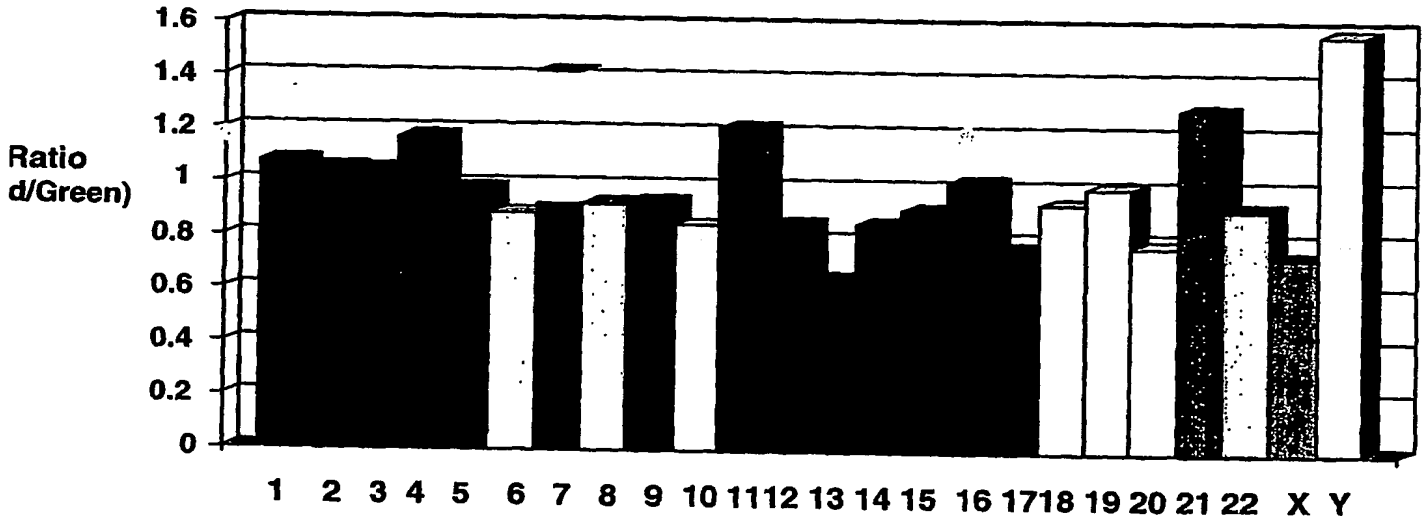
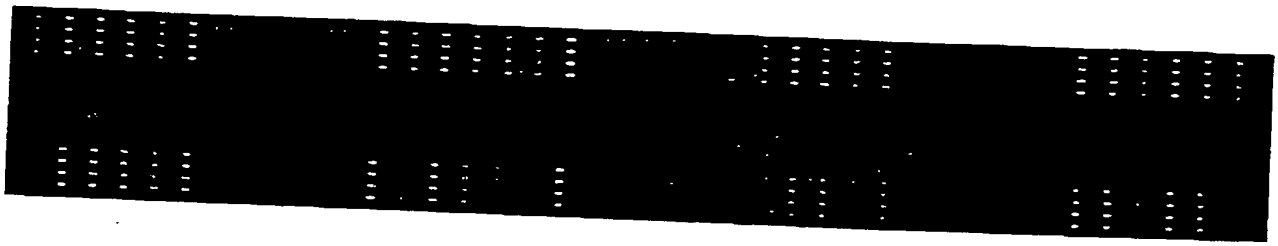

Ratio
d/Green)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 2 3 4 5 6 7 8 9 10 1112 13 14 15 16 1718 19 20 21 22 X Y

Figure 6



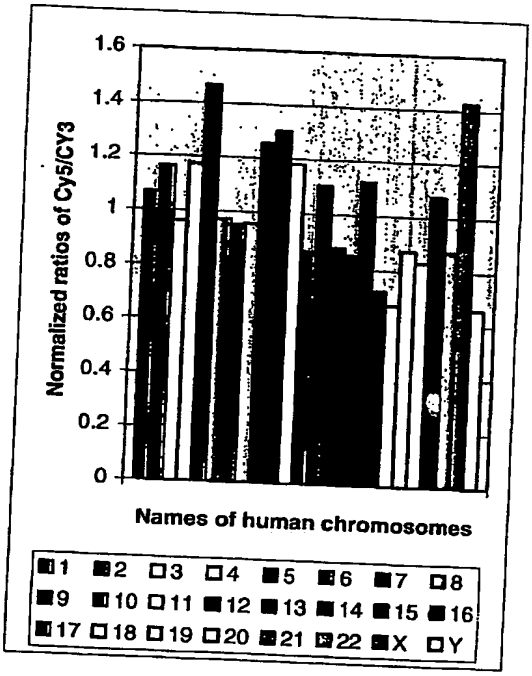

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Normalized ratios of Cy5/CY3
Names of human chromosomes
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
17 18 19 20 21 22 X Y

Figure 7

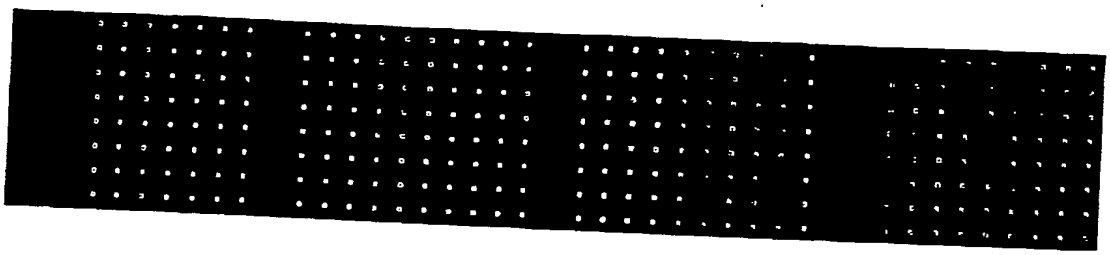
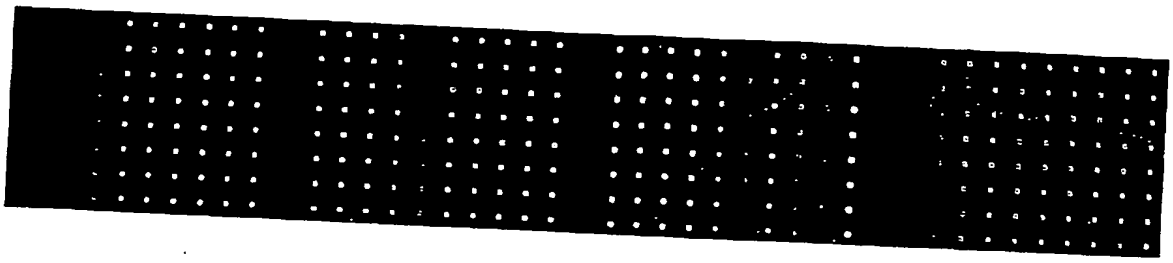


Figure 8



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